PhD Thesis

Interactions between tumour and natural killer cells in primary and secondary liver cancer

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Declaration

I, Nicholas James Wilson Easom, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:_____

Date:_____

Abstract

Natural killer (NK) cells are implicated in tumour surveillance and control in a number of settings. The liver contains large numbers of NK cells and hepatocellular carcinoma (HCC) has been shown to express various ligands that permit interactions with NK cells, some of which have been shown to have prognostic significance. Similar prognostic associations have been shown for colorectal cancer, a tumour which commonly metastasizes to the liver. However the mechanistic underpinning of these effects is unclear.

This thesis describes a survey of soluble NKG2D ligand expression in patients with HCC caused by chronic hepatitis B (CHB), patients with CHB without cancer, and healthy controls. ULBP1 was found to be raised in HCC patients; where it was associated with poor survival, and in active CHB; where it was associated with hepatitis B viral load. ULBP1 was not seen in secondary liver tumours, suggesting that this protein may be useful as a biomarker of severe disease or to monitor treatment response. The other NKG2D ligands MICA, MICB, ULBP2 and ULBP3 were not found to be elevated in patient serum. Soluble NKG2D ligands did not affect NKG2D expression by circulating NK cells.

Tumour infiltrating NK cells have the potential to be important effector cells, but are functionally defective. This work builds on recent advances in the understanding of liver-resident NK cells to characterise the functional defect in primary and secondary liver cancers, using human *ex vivo* intrahepatic and tumour-infiltrating NK cells. Using an *in vitro* model of HCC we have investigated the mechanisms for this defect and examine the potential for IL-15 to restore NK cell cytotoxicity and cytokine production. *Ex-vivo* tumour-infiltrating NK cells had reduced NKG2D expression, IFNγ production and degranulation potential compared with paired intrahepatic NK cells, but maintained expression of NKp46. We were able to recapitulate this

phenotype by co-culture with an HCC cell line, but were not able to protect NK cells from NKG2D downregulation and functional inhibition by NKG2D blockade. However, IL-15 was able to restore function after HCC exposure. This model may serve as an *in-vitro* assay for future therapeutics targeting tumour-infiltrating NK cells.

Impact statement

Hepatocellular carcinoma (HCC) is the third largest cause of cancer death in humans, responsible for over 750,000 deaths annually. The conventional cancer treatment modalities are largely ineffective in this disease. Surgical cure is possible, but only where HCC is diagnosed early, which is rare. Cytotoxic chemotherapy is ineffective, as is radiotherapy. Sorafenib, the single licensed drug for HCC can extend life for 2-3 months, but does not induce objective responses.

Recent advances in immunotherapy have shown promise in HCC. Blockade of programmed-death 1 (PD1) allows T cells to respond to tumours, and in HCC has shown 20% response rates. We have explored interactions between HCC and natural killer (NK) cells, a lymphocyte present in large numbers in the liver, in an attempt to engage their cytotoxic potential against HCC.

Patients with HCC were found to have elevated circulating levels of ULBP1, a ligand for the activating NK cell receptor NKG2D. This protein did not affect circulating NK cells, but was associated with poor prognosis in HCC. This finding could be utilised clinically either to screen for HCC in at-risk patients, or as a prognostic marker in HCC. It might also be important in the selection of future immunotherapies, as NK cells transfected with NKG2D chimeric antigen receptors are currently being investigated for HCC, and the circulating NKG2D ligand environment may be an important consideration in selecting personalized treatment.

Unexpectedly, circulating ULBP1 was also found in patients with chronic hepatitis B that required treatment. There is a need in low-middle income countries for a simple, inexpensive test that can substitute for the current, expensive and poorly available investigations required to assess individuals

with hepatitis B infection. Development of the current ELISA into a lateral-flow assay might greatly improve access to hepatitis B assessment and therefore treatment – a key goal of the 2016 WHO global strategy for viral hepatitis.

We have also examined the phenotype and function of NK cells in liver tumours and healthy liver tissue from the same donors, and using a hepatocellular carcinoma cell line have been able to model the inactivation of human intrahepatic NK cells interacting with HCC. This platform allows the testing of potential drugs to re-activate NK cell anti-tumour function. IL-15 is one such agent, and modified or targeted forms of IL-15 could be rapidly tested in this model before progressing to animal models or human trials. The activity of other classes of drugs, such as receptor blocking antibodies, can also be tested in this model.

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ADCC	antibody-dependent cell cytotoxicity
AFP	alphafetoprotein
ALT	alanine transaminase
BIM	BCL2-interacting mediator of cell death
BSA	bovine serum albumin
CAR	chimeric antigen receptor
cccDNA	covalently closed circular DNA
CCL	CC chemokine ligand
CD	cluster of differentiation
CHB	chronic hepatitis B
CMV	cytomegalovirus
c-Myc	cellular myelocytomatosis oncogene
CRC	colorectal cancer
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAMP	damage-associated molecular pattern
DAP10	DNAX-activating protein of 10kD
DAP12	DNAX-activating protein of 12kD
DEN	diethylnitrosamine
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
EASL	European Association for the Study of the Liver
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
EU	European Union
FBS	fetal bovine serum
GPI	glycosylphosphatidylinositol
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBSP	hepatitis B spliced protein
HBV	hepatitis B virus
HBx	hepatitis B x protein

HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HER2	human epidermal growth factor receptor 2
HLA	human leukocyte antigen
HPC	haematopoietic precursor cell
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IL2R	IL-2 receptor
IU	international units
KIR	killer cell immunoglobulin-like receptor
LAG3	Lymphocyte activation gene 3
LFA1	lymphocyte function-associated antigen 1
MAGE-1	melanoma-associated antigen 1
MAIT	mucosal-associated invariant T cell
mbIL-15	membrane bound IL-15
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MRC	Medical Research Council
mRNA	messenger RNA
NASH	non-alcoholic stator-hepatitis
NK	natural killer
NKG2A	natural killer group 2 receptor A
NKG2C	natural killer group 2 receptor C
NKG2D	natural killer group 2 receptor D
NKp46	natural killer cell p46 related-protein
NOD	non-obese diabetic
NTCP	sodium taurocholate cotransporting peptide
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PD1	programmed-death 1
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
PLZF	promyelocytic leukaemia zinc finger
RNA	ribose nucleic acid
RPMI	Roswell Park Memorial Institute medium

Scid	severe combined immunodeficiency
SMAD3	mothers against decapentaplegic homolog 3
Syk	spleen tyrosine kinase
TACE	trans-arterial chemo-embolisation
Tim-3	T cell immunoglobulin and mucin domain 3
TGF	transforming growth factor
TNF	tumour necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
ULBP	unique long 16 binding protein
WHO	World Health Organisation
ZAP70	Zeta chain-associated protein kinase 70

Chapter 1 Introduction

1.1 Biology of Natural Killer cells

Natural Killer (NK) cells, so called because of their ability to display cytotoxicity without prior sensitisation (natural killing), are innate lymphocytes present in blood and other tissues (Kiessling et al. 1975; Caligiuri 2008; Spits et al. 2013; Eberl et al. 2015). Like other lymphocytes they are derived from CD34+ haematopoietic precursor cells (HPC), predominantly from the bone marrow (Di Santo 2006), although CD34+ precursors giving rise to NK cells have been seen in secondary lymphoid tissue including thymus and gut-associated lymphoid tissue, peripheral blood, cord blood and decidua (Vacca et al. 2006; Bozzano et al. 2017). Within the last 10 years it has become apparent that NK cells are a subset of the innate lymphoid cells (ILCs) (Spits et al. 2016; Klose & Artis 2016). There are believed to be five major ILC populations, which arise from the same common lymphoid precursor and share canonical transcription factors and cytokine production with T cell populations: NK cells driven by Eomes and secreting IFNγ are analogous to CD8 T cells; ILC1 cells driven by T-bet and secreting IFNγ are analogous to CD4 T_H1 T cells; ILC2 cells driven by GATA-3 and secreting IL-4, IL-5 and IL-13 are analogous to CD4 T_H2 T cells; and two populations of ILC3 cells (differentiated by their expression of NKp46) driven by RORγt and secreting IL-17 and IL-22 are analogues of CD4 T_H17 and T_H22 T cells (Eberl et al. 2015). As might be implied by this classification, the ILCs are developmentally distinct from NK cells, arising from the common helper innate lymphoid progenitor (CHILP), as a first stage in differentiation from the early innate lymphoid progenitor (Zook & Kee 2016). However, murine ILC1 precursors are indistinguishable from NK cell precursors without fate mapping of the transcription factor PZLF (Constantinides et al. 2015). Mature ILCs are CD3 negative lymphocytes residing in tissues, are CD127 (IL7 receptor α) positive and do not express CD56 (Spits et al. 2016). In mice, fate mapping studies have demonstrated that there is plasticity of ILC

phenotypes, with IL-12 able to induce transdifferentiation of both ILC2s and ILC3s into ILC1 cells (Vonarbourg et al. 2010; Ohne et al. 2016; Lim et al. 2016). Conversely, ILC1s can be induced to transdifferentiate into ILC2s in response to IL-4 and into ILC3 cells in response to IL1β and IL-23 (Bal et al. 2016; Bernink et al. 2015). NK cells and ILC1 cells have some similarities, particularly in mice where they are both NK1.1+, NKp46+ cells that secrete IFNγ. ILC1s have important roles in colitis, both infectious and inflammatory (Abt et al. 2015; Buonocore et al. 2010; Bernink et al. 2013). ILCs may have important roles in responses to cancer. ILC1-like cells have been seen in a murine cancer model, although their specific role remains unclear (Dadi et al. 2016). In cultures of tumour-infiltrating lymphocytes expanded with high doses of IL-2, an innate lymphoid population suppressed anti-tumour T cell proliferation and had a transcriptional profile overlapping with ILCs and NK cells (Crome et al. 2017). Whether there is an *in-vivo* equivalent of this cell remains uncertain.

A five-stage process of NK cell maturation has been proposed (Freud et al. 2014) (Figure 1.1), giving rise to CD56bright (stage 4) and CD56dim (stage 5) NK cells in the periphery. However, recent work suggests that an alternative, transcriptionally distinct, CD34+ precursor may leave the bone marrow and differentiate directly into mature NK cells (and T cells) in the periphery in the setting of inflammatory disease, bypassing the maturation that traditionally occurs in the lymph node (Bozzano et al. 2015). It may be that phenotypically similar NK cells can arise from different developmental pathways (Bozzano et al. 2017). IL-15 is considered the most important cytokine in NK homeostasis. It promotes NK cell differentiation, proliferation and survival (Carson, Giri, Lindemann, Linett, Ahdieh, Paxton, Anderson, Eisenmann, Grabstein & Caligiuri 1994a; Carson et al. 1997; Mrózek et al. 1996). Expression of CD122, the beta chain shared by the IL-2 and IL-15 receptors in stages 2-3, allows immature NK cells to respond to IL-15, transpresented by dendritic cells in the lymph node, to differentiate into

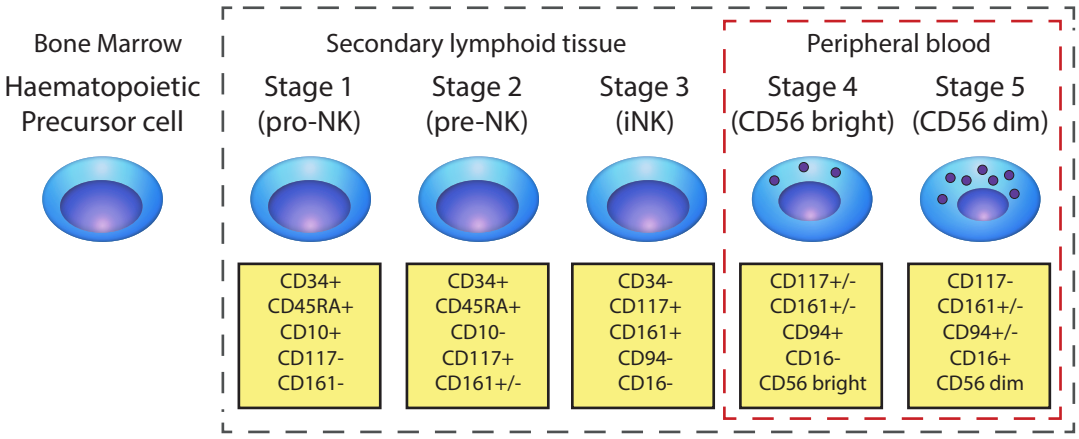

Figure 1.1 Schematic of NK cell development.
Adapted from Freud *et. al.* 2014. NK cells arise from the common lymphoid progenitor in the bone marrow. Early stages of NK cell development occur in the bone marrow, and are associated wth the expression and then loss of CD34. The final stages of NK cell development may occur in lymphoid tissue or in the peripheral blood. In the final stage of development NK cells express CD16, the Fc gamma receptor III that is responsible for antibody dependent cell cytotoxicity.

CD56bright NK cells (Freud et al. 2005; Mrózek et al. 1996; Mattei et al. 2001).

Both CD56bright and CD56dim NK cells may be found in peripheral blood and other tissues (Björkström et al. 2016). Their activity is determined by a range of germline-encoded receptors that give activatory and inhibitory

signals (Long et al. 2013). The balance of these signals determines activation, leading to the major effector functions of cytotoxicity by release of lytic granules containing perforin and granzymes, and production of the inflammatory cytokines interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα), as well as ligation of death receptors by expression of death ligands such as TRAIL (Lanier 2008b). The expression of each of the various NK cell receptors produces remarkable phenotypic diversity among the NK cell population that is only beginning to be understood (Horowitz et al. 2013). NK cell activatory and inhibitory receptors and their ligands are species-specific, and many have undergone rapid evolution (Parham & Moffett 2013; Carrillo-Bustamante et al. 2016). The human NK cell receptors and their ligands are shown below (Figure 1.2). The ligands of some receptors remain unknown, the most recent discovery is the ligand for NKp46, shown to be properdin, or complement factor P (Narni-Mancinelli et al. 2017). These receptor-ligand interactions occur in a highly organised structure known as the immune synapse (Davis et al. 1999). Contact, granule polarisation, and degranulation are complex processes requiring multiple signals, typically an adhesion signal via the integrin LFA1 and an activatory signal via the antibody-dependent cell cytotoxicity (ADCC) receptor CD16, or other activatory receptors such as NKG2D, 2B4 or NKp46, often in concert (Bryceson et al. 2006). Super-resolution imaging of the immune synapse and underlying cytoskeletal remodelling has shown that different activating receptors have different potency, for example that NKp46 is unable to rearrange cortical actin to allow granule exocytosis without an additional

Activating receptors

Receptor	Ligand
CD16	Immunoglobulin G
NKG2D	MICA/B, ULBP1-6
NKG2C	HLA-E
NKp46	Complement factor P
NKp30	B7H6, BAT3
NKp44	Viral haemaglutinins
NKp80	AICL
DNAM-1	PVR, Nectin 2
CRACC	CRACC
KIR-S	HLA-C
2B4	CD48

Inhibitory receptors

Receptor	Ligand
KIR-L	HLA-C, B, A
NKG2A	HLA-E
LAIR-1	Collagens
KLRG-1	Cadherins
SIGLECs	Sialic acid
LILRB1	HLA class I
TIGIT	PVR, Nectin 2, CD113

Adhesion receptors

Receptor	Ligand
CD2	CD58
β1 integrins	VCAM-1
β2 integrins	ICAM1, ICAM2

Figure 1.2 Summary of the human NK cell receptor repertoire. Adapted from Vivier *et. al.* 2008 and Vivier *et. al.* 2011. Tables to illustrate the NK cell receptors available for cell-cell contacts. These lists are not exhaustive and cytokine and chemokine receptors are not depicted. BAT3, HLA-B-associated transcript 3; AICL, activation-induced C-type lectin; DNAM-1 (also known as CD226), DNAX Accessory Molecule-1; PVR (also known as CD155), poliovirus receptor; CRACC, CD2-like receptor-activating cytotoxic cell; KIR, killer immunoglobulin-like receptors; LAIR, Leukocyte-associated immunoglobulin-like receptor; KLRG1, killer cell lectin-like receptor G1; SIGLEC, sialic acid binding immunoglobulin-like lectins; LILRB1, Leukocyte immunoglobulin-like receptor subfamily B member 1; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule.

signal provided via LFA1, whereas NKG2D or CD16 signalling alone is able to induce actin rearrangement (A. C. N. Brown et al. 2012).

As well as responding to ligands expressed on cell surfaces, NK cell activity is modulated by a number of cytokines. The related cytokines IL-2 and IL-15 are important in NK cell homeostasis. IL-2 induces NK cell proliferation

(T. K. Yu et al. 2000; Warren et al. 1996), and at high levels can be used to generate lymphokine-activated killer cells (LAK), which have been used in early cancer immunotherapy (Rosenberg et al. 1985). IL-12 is the most important signal driving production of IFNγ, usually supplemented by additional signals, such as IL-1, IL-2, IL-15, IL-18, or CD16 or NKG2D engagement (Parihar et al. 2002; Ferlazzo et al. 2004; Caligiuri 2008). The combination of T cell-derived IL-2 and IL-12, produced by dendritic cells and cells of the monocyte/macrophage lineage, is a potent stimulus for IFNγ production, which in turn activates antigen presenting cells (APC). IL-12, IL-15 and IL-18 also antagonise the inhibitory effect of transforming growth factor beta (TGF-β) (J. Yu et al. 2006). IL-15 also drives cytotoxicity, and in mice can contribute to anti-tumour immunity (Sutherland 2006).

Killer immunoglobulin-like receptors provide an important control on NK cell function and are a major mechanism by which “missing self” behaviour is generated. A key feature of this system is diversity of both receptors and their HLA class I ligands, across species, individuals, cells and genes (Parham & Moffett 2013). Simian primates have diversified KIR genes from the founder gene *KIR3DL*, whereas cattle have an independent KIR system diversified from *KIR3DX*, which is inactive in simians (Guethlein et al. 2007). Mice use a diversified set of LY49 receptors, a genetically distinct set of molecules related to calcium-dependent lectins, which bind a separate site on MHC class I molecules (Natarajan et al. 2002). In humans there are three lineages of KIRs that bind HLA class I, lineage I KIRs bind HLA-G, lineage II KIRs bind

the A3/11 epitope of HLA-A or the Bw4 epitope of HLA-A and HLA-B. lineage III KIRs bind either the C1 or C2 epitopes of HLA-C. The KIR genes are arranged at the KIR locus of chromosome 19 into a centromeric region and a telomeric region. Two major haplotypes, A and B, are seen in human populations, whereas chimpanzees have only A haplotypes (Pyo et al. 2010). The A haplotypes have a fixed gene content, and predominantly encode inhibitory KIRs that bind HLA class I as above, whereas B haplotypes are characterised by the presence of KIR genes that have little or no binding of HLA class I, such as KIR2DS2, KIR2DS3, and KIR2DS5, and may have variable numbers of KIR genes (Uhrberg et al. 1997). By reassortment a single locus may contain A or B motifs but all haplotypes containing B motifs are considered B haplotypes. A dynamic equilibrium maintains both haplotypes within human populations, with A haplotypes protecting from infection but increasing the risk of disorders of placentation, where NK cells play an important role (Khakoo et al. 2004; Nakimuli et al. 2015). Rapid evolution at this locus has been demonstrated by the study of isolated human populations, including the observation of the emergence of novel KIR genes by recombination, particularly where HLA class I variation is limited due to founder effects and evolutionary bottlenecks (Gendzekhadze et al. 2009; Nemat-Gorgani et al. 2014). This demonstrates the importance of the maintenance of KIR diversity at a population level (Manser et al. 2015).

During NK cell maturation, NK cells will express inhibitory killer cell immunoglobulin-like receptors (KIR) in a seemingly stochastic fashion until they express one that interacts with self-MHC, present on almost every cell in the body. This process is known as NK cell licencing, and allows for potent cytotoxicity when the resultant tonic inhibition is absent (S. Kim et al. 2005; Anfossi et al. 2006). Maintenance of KIR expression is dependent on DNA methylation, resulting in a variety of NK cell clones *in vivo*, each with stable KIR expression (Chan et al. 2003). Recent work suggests that in individuals with certain HLA class I haplotypes, NKG2A-HLA-E interactions can

substitute for KIR in NK cell licencing (Horowitz et al. 2016). NK cell licencing can be exploited in allogeneic NK cell transfer, where transfer into an HLA-mismatched recipient removes this functional inhibition (Ruggeri et al. 2002). NK cells from patients who do not express HLA class I remain “uneducated” and are unable to lyse class I-deficient targets (Zimmer et al. 1998; Furukawa et al. 1999). In normal individuals all cytotoxic NK cells express either a KIR specific for self-HLA class I, or NKG2A (Valiante et al. 1997).

The biological consequences of NK cell activation vary depending on the setting and the other cells involved in the interaction, and in many settings, despite their name, cytotoxicity may be less important than cytokine production (Vivier et al. 2008). As effectors, NK cells have important roles in tumour surveillance and immunity (see below), and the control of viral infection. The evidence for the importance of NK cells in viral infection in humans comes from four main sources: clinical observations in NK cell-deficient humans, observations that certain NK cell receptors or ligands confer susceptibility or protection to certain viruses, animal models, and the imprint of NK cell-mediated evolutionary pressure on viral infections of humans (S.-H. Lee et al. 2007; Jost & Altfeld 2013). Humans with absent or defective NK cells are rare, but suffer severe and recurrent herpesvirus infections, and also seem to be disproportionately affected by papillomavirus and virally-driven malignancy (Biron et al. 1989; Orange 2013) Functional NK cell deficiency due to mutation in the *FCGR3A* gene, which encodes CD16, is associated with susceptibility to herpesvirus infections and viral respiratory infections (de Vries et al. 1996; Jawahar et al. 1996). KIR genotyping studies have shown multiple associations between NK cell receptor repertoire and disease outcome, including the protective effect of the activating KIR3DS1 in the presence of HLA Bw4-80I in HIV (Bashirova et al. 2011), the protective effect of KIR2DL3 in the presence of HLA-C1 in HCV (Khakoo et al. 2004) and the recently described protective effect of KIR2DL3 in HBV infection (Di Bona et al. 2017). Murine models have demonstrated the importance of NK

cells in immune responses to a range of viruses (Biron et al. 1999; Lanier 2008a). In an early murine cytomegalovirus (MCMV) infection model, NK depleted mice had higher viral titres and more severe pathology (Bukowski et al. 1983). Subsequent work showed that the activating receptor Ly49H is critical for NK cell responses to MCMV (M. G. Brown et al. 2001). More recently, Joseph Sun et al demonstrated that following MCMV infection, a population of Ly49H+ NK cells expanded rapidly after infection in a DAP12-dependent manner, and was maintained for several weeks. These NK cells expanded following infection demonstrated increased IFNγ production and degranulation on stimulation compared with naïve Ly49H+ NK cells, and were protective when adoptively transferred into DAP12-/- mice infected with MCMV (J. C. Sun et al. 2009). These observations are complimented by human data showing complex interactions between human CMV and NK cells (Di Bona et al. 2014). CMV can drive NK cell differentiation and expansion (Lopez-Vergès et al. 2011; Foley, Cooley, Verneris, Pitt, et al. 2012), although similar effects have been seen in other viral infections (Björkström et al. 2011). For example, NK cells have been shown to be important in immune responses to influenza in mice and humans (Gazit et al. 2006; Draghi et al. 2007). Furthermore, murine models have demonstrated a role for NK cells in regulation of immune responses to control immune pathology in lymphocytic choriomeningitis virus (Waggoner et al. 2011). In this model, NK cell depletion protected against excess immune pathology in medium dose infection, but led to high mortality in high dose infection despite a reduction in viral load. Reduced survival in high dose infection was CD4 T cell-dependent and NK cells were shown to be able to eliminate activated CD4 cells to control immune pathology at the expense of viral control. Subsequently similar regulatory function was demonstrated in human viral infection (Peppa et al. 2013). Additionally, many viral genomes encode proteins that help them evade NK cell responses, either by sequestration of activating ligands for NK cells or by mimicking inhibitory ligands (Cosman et

al. 2001; Wilkinson et al. 2008; Lisnić et al. 2010). Finally, there is evidence of KIR “footprints” in the sequences of HIV-1, suggesting adaptation of the virus to more strongly bind inhibitory KIR and thereby prevent NK cell activation (Alter et al. 2011). However, other authors have suggested that many of these polymorphisms cannot be explained by KIR pressure as the required KIR-HLA genotype is too scarce within the population (Elemans et al. 2017). In any case, several lines of evidence demonstrate the importance of NK cells in immune responses to viruses.

NK cells also have emerging and complex interactions with other arms of the immune response. NK cells can delete T cells in chronic viral infection to modulate immune pathology (Waggoner et al. 2011; Peppa et al. 2013). They can stimulate monocytes to secrete tumour necrosis factor alpha (TNFα) (Dalbeth et al. 2004), and in mice are capable of editing the dendritic cell (DC) population in a perforin-dependent manner to enhance tumour-specific T cell responses (Morandi et al. 2012). In systemic infection NK cells can also produce IL-10 in response to IL-12, inhibiting further IL-12 production by DCs, acting as a feedback loop to modulate inflammation (Perona-Wright et al. 2009). In this way NK cells are thought of as bridging the divide between innate and adaptive immunity (Vivier et al. 2011).

NK cell memory has emerged in recent years as another facet of NK cell biology to blur the distinction between innate and adaptive (Cerwenka & Lanier 2016). In mice, NK cell memory has been seen in response to haptens (O'Leary et al. 2006; Paust et al. 2010) and viruses (J. C. Sun et al. 2009; Gillard et al. 2011). The hapten-induced contact hypersensitivity (CHS) model used by the von Andrian group, based on painting 2,4 dinitro-1-fluorobenzene or oxalozone onto the ears of mice and measuring ear thickness on challenge and re-challenge, may provide the best evidence of true NK cell memory behaviour. RAG2 knockout, IL2 receptor γ knockout mice (T, B and NK cell deficient) that receive adoptively transferred NK cells

from hapten-sensitised mice display much stronger CHS reactions than those that receive NK cells from naïve mice, and NK cells transferred 4 months after sensitisation induced stronger CHS than NK cells transferred 24 hours after sensitisation. This behaviour was replicated on transplant of liver but not spleen tissue, and congenically marked NK cells were seen in ear tissue following liver transplant and re-challenge with the hapten to which they were previously exposed, but not to an alternative hapten. Similar behaviour was seen following subcutaneous injection of viral antigens, and liver transplant from influenza VLP sensitized mice protected naïve mice from the corresponding influenza strain. Anti CXCR6 antibody administered to wild-type or RAG knockout mice significantly reduced the CHS response on re-challenge either with haptens or viral antigens, and CXCR6 knockout mice did not induce CHS on NK cell adoptive transfer. Finally, sensitised liver NK cells were able to specifically kill haptenated B cell targets, but importantly, were shown to have equivalent activity to splenic or non-sensitised NK cells against MHC knockout targets (Paust et al. 2010). This is the best evidence for NK-mediated, long-lived, antigen-specific recall responses, which justify use of the term “memory”, although the molecular basis for these responses remains uncertain (Paust & Andrian 2011; Rölle et al. 2013). More recently NK cells from SHIV infected and SHIV vaccinated rhesus macaques have been shown to lyse peptide-pulsed dendritic cells in an antigen specific manner using a mechanism dependent of both NKG2C and NKG2A (Reeves et al. 2015). Whether the same mechanisms are available to human NK cells remains unclear, although their potential applications are already generating interest (Cerwenka & Lanier 2016).

In response to the cytokines IL-12, IL-15 and IL-18, so-called “cytokine-induced memory NK cells” have been generated, which have high levels of perforin and granzyme and on restimulation with IL-2 after adoptive transfer, can exert a long-lived anti-tumour effect (Cooper et al. 2009; Ni et al. 2012). This combination of cytokines causes demethylation of the conserved non-

coding sequences upstream of the interferon gamma locus, which may explain the increased activity of these cells (Luetke-Eversloh et al. 2014). However, other authors have suggested that this is the consequence of the poor differentiation of NK cells in mouse houses compared to in free-living mice, and that “cytokine-induced memory NK cells” may reflect normal NK cell development (Boysen et al. 2011; Riley & Viney 2011). In humans there is no evidence for antigen-specific recall responses, but the expanded subset of NKG2C+ CD57+ NK cells driven by CMV reactivation in the transplantation setting has been labelled “memory-like” (Lopez-Vergès et al. 2011; Foley, Cooley, Verneris, Curtsinger, et al. 2012). Interestingly, the same demethylation pattern at the interferon gamma locus seen in cytokine-primed NK cells was seen stably imprinted in NKG2C hi NK cells from HCMV+ donors (Luetke-Eversloh et al. 2014). More recent work has shown that this population is characterised by the epigenetic silencing of the transcription factor PLZF, and based on their genome-wide DNA methylation pattern, the term “adaptive NK cell” has been used (J. Lee et al. 2015; Schlums et al. 2015).

1.2 NKG2D and its ligands

NKG2D is one of the C-type lectin-like receptors (Wu 1999; P. Li et al. 2001). Unlike NKG2A and NKG2C, which form heterodimers with CD94, NKG2D forms a homodimer. In humans, each homodimer recruits two DAP10 (DNAX-activating protein of 10kD) dimers and signals via their YINM domains to give an activatory signal via Vav1 and phosphoinositide 3-kinase (PI3K), whereas in mice, NKG2D can signal via DAP10 or DAP12, which contains canonical ITAM domains, to signal via Syk and ZAP70 (Lanier 2015). NKG2D is expressed on NK cells and some T cells (Bauer et al. 1999). It is highly conserved in humans and other mammals, but the ligands are numerous and variable (Eagle & Trowsdale 2007). Regulation of NKG2D activity is at the level of the ligands. In humans there are eight known

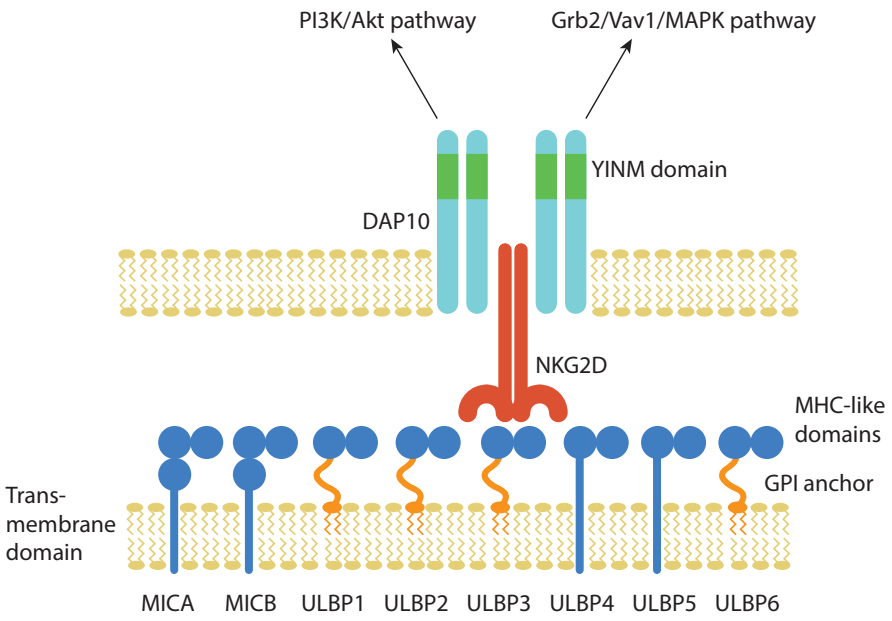

Figure 1.3 Human NKG2D and NKG2D ligands.
MICA and MICB are distant homologues of MHC class I and have three domains that resemble the three domains of the alpha chain of HLA class I, but with no capacity for peptide binding. ULBP1-6 retain the alpha-1 and -2 domains only, and ULBP1, 2, 3 and 6 are not transmembrane proteins, but are tethered to the cell by GPI anchors.

Akt, Protein kinase B; Grb2, growth factor receptor-bound protein 2; MAPK, mitogen activated protein kinase; GPI, glycosylphosphatidylinositol; MIC, MHC class I polypeptide-related sequence; ULBP, unique long 16 binding protein.

ligands – MICA, MICB, ULBP1-6, all distant homologues of MHC class I (Figure 1.3) (Raulet et al. 2013), that are upregulated in response to cellular stresses including DNA damage and viral infection (Gasser et al. 2005; Ward et al. 2009). In the mouse, there are five isoforms of retinoic acid early inducible-1 (RAE-1α-ε), three isoforms of H60, and the high affinity murine UL16-binding protein-like transcript 1 (MULT1). The murine ligands all have two extracellular domains, orthologous to the ULBPs, with RAE-1 proteins being GPI anchored, whereas H60 and MULT1 are transmembrane proteins (Eagle & Trowsdale 2007).

The biological reason for and purpose of this multitude of ligands is unclear, but one reason must be gene duplication to counteract the sequestration of NKG2D ligands by specific proteins synthesised by viruses such as CMV (Slavuljica et al. 2011). Another product of ligand multiplicity is that it allows the expression of NKG2D ligands that engage NKG2D with differing affinities, with different functional consequences (Raulet et al. 2013; Spear et al. 2013; Zuo et al. 2017).

NKG2D is known to be important in immune surveillance of cancer and tumour immunity (N. Guerra et al. 2008). NKG2D ligands have been found on various cancers, often associated with an improved prognosis (McGilvray et al. 2009; Cho et al. 2014; Kamimura et al. 2012). Conversely the shedding of soluble forms of NKG2D ligands from tumours has been proposed as a mechanism of immune escape (Groh et al. 2002; Mincheva-Nilsson & Baranov 2014). These ligands are predominantly released by proteolytic shedding from the cell surface (Salih et al. 2002; Waldhauer & Steinle 2006), although the glycosylphosphatidylinositol (GPI)-anchored ligands can be released as part of exosomes (Fernández-Messina et al. 2010). Soluble splice variants of some of the NKG2D ligands are known, but their biological significance is unclear (Bacon et al. 2004; Gavlovsky et al. 2016). Soluble NKG2D ligands are generally thought to act by NKG2D downregulation,

impairing the ability of NK cells to respond (Groh et al. 2002; Song et al. 2006). However, in some murine systems soluble NKG2D ligands act differently, blocking a tonic NKG2D downregulation due to ligands expressed on the surface of parenchymal cells, resulting in improved NK cell killing (Oppenheim et al. 2005; Deng et al. 2015) (Figure 1.4). It is unclear whether a similar phenomenon might occur in humans – downmodulation of NKG2D function in response to surface-expressed NKG2D ligands has been associated with a replacement of DAP10 signalling with DAP12 signalling, a transition which is unavailable to human NK cells (Coudert et al. 2005).

1.3 Other activating receptors

Other groups of activation receptors also have important roles in NK cell function. The natural cytotoxicity receptors NKp30, NKp44 and NKp46 are among the best studied, particularly NKp46 and its murine ortholog, NCR1 (Sivori et al. 1997; Biassoni et al. 1999). NKp46 is expressed by mature NK cells and certain ILC subsets (Narni-Mancinelli et al. 2011; Eberl et al. 2015). It signals via FCεRI and CD3ζ (Westgaard et al. 2004) and has been associated with various ligands including neuraminidase of influenza and other viruses, adhesins of *Candida glabrata* but not other Candida species, an unknown ligand of *Fusobacterium nucleatum* and vimentin, a protein synthesised by *Mycobacterium tuberculosis*-infected cells (Kruse et al. 2014). The interactions with viral haemaglutinins are mediated via sialic acid residues on NKp46 (Mandelboim et al. 2001). The same group has shown that NKp46 may also have a role in tumour immunity, and is involved in killing tumour cells, suggesting there may be an undiscovered ligand associated with malignancy (Glasner et al. 2012). More recently complement factor P (CFP) has been found to be a ligand for NKp46 and the CFP-NKp46 interaction was shown to be protecting in *Neisseria meningitidis* infection in a mouse model (Narni-Mancinelli et al. 2017). The molecular basis for the binding of NKp46 with this diverse range of ligands remains unclear

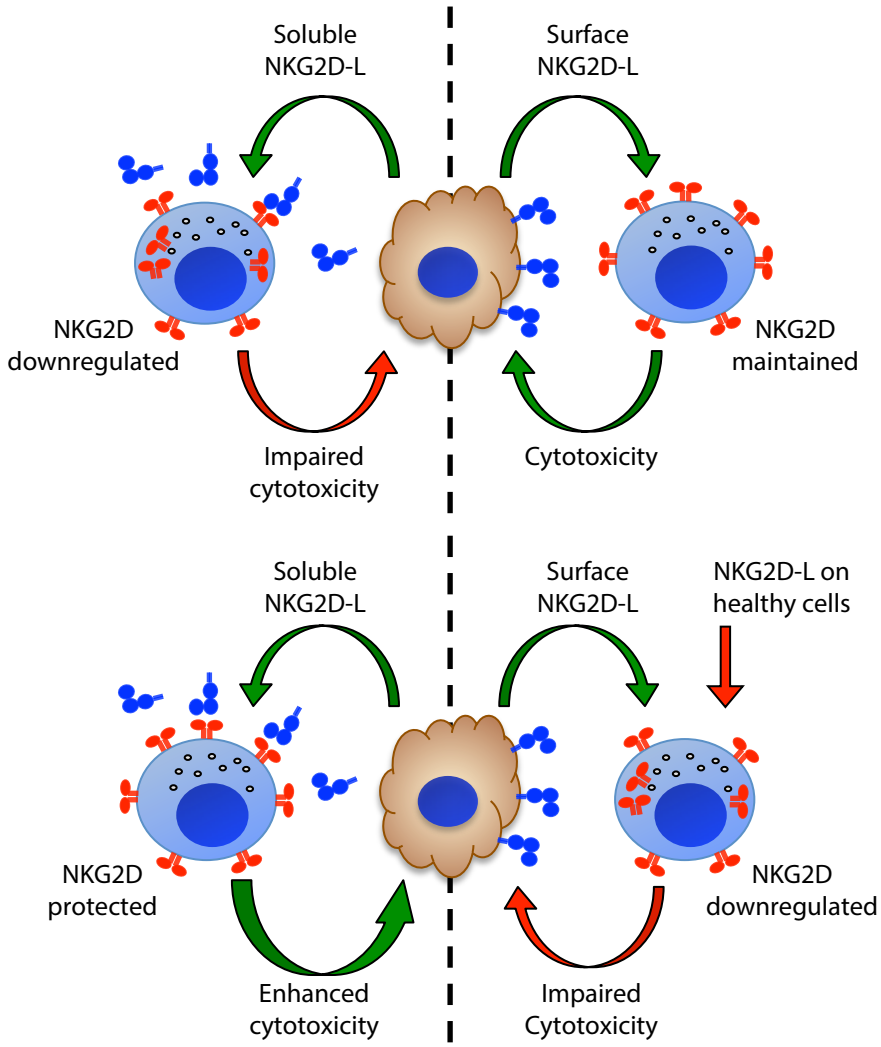

Figure 1.4 Competing views of the role of soluble NKG2D ligands in cancer

Adapted from Mondelli 2012 and Deng et al 2015. The traditional view has been that NK cells recognise surface NKG2D ligands on tumour cells, but that soluble NKG2D ligands engage NKG2D, causing its downregulation, thereby hiding tumour cells from detection by NK cells (top). Recent experiments in a mouse melanoma model (Deng et al 2015) have described a system whereby NKG2D is tonically downregulated by exposure to NKG2D ligands on resting, healthy cells. The soluble form of MULT1, a murine NKG2D ligand, was able to maintain NKG2D expression and enhance NK cell killing of melanoma in a dominant fashion. We will investigate whether a similar mechanism may be relevant in human cancer.

(Kruse et al. 2014). Interestingly, overexpression of NKp46 using a flox-cre system increased the activity of natural killer cells against influenza and tumour metastases in two mouse models (Glasner et al. 2017).

DNAX accessory molecule-1 (DNAM-1) is a transmembrane glycoprotein of the immunoglobulin superfamily. Its main ligands are Nectin-2 and the poliovirus receptor (PVR), a nectin-like molecule (Tahara-Hanaoka et al. 2004). DNAM-1 is expressed on both NK and T cells, and has close associations with both the cytoskeleton and the adhesion molecule LFA-1, suggesting it may be important in immune synapse formation (de Andrade et al. 2014). DNAM-1 appears to cooperate with other activating ligands in the killing of target cells, and but NKG2D ligands and DNAM-1 ligands may be induced by DNA damage, leaving them well placed to act together in antiviral and anti-tumour responses (El-Sherbiny et al. 2007; Carlsten et al. 2007; Cerboni et al. 2014).

1.4 Hepatitis B virus and Chronic Hepatitis B

The molecular biology of hepatitis B is reviewed in Seeger and Mason 2015 (Seeger & Mason 2015). In brief, the virion contains a relaxed circular DNA genome of 3.2 kb with the DNA polymerase covalently linked (Kaplan et al. 1973; Summers et al. 1975). Viral tropism is specific for hepatocytes, and entry requires the bile acid transporter, sodium taurocholate cotransporting peptide (NTCP), as the viral receptor (Yan et al. 2012). On entry into the hepatocyte nucleus, synthesis of the positive strand of the DNA genome is completed by the viral polymerase to form a covalently closed circular DNA genome (cccDNA), establishing a lifelong reservoir of HBV infection (Newbold et al. 1995). This structure is long-lived within the nucleus and forms the template for mRNA synthesis by host RNA polymerase (Bock et al. 2001). Seven major proteins are synthesised from the four overlapping open reading frames (ORF) (Moolla et al. 2002) (Figure 1.5). Polymerase is a reverse transcriptase, RNAse H and primer (Hu & Seeger 2015). Core forms

the viral nucleocapsid (Crowther et al. 1994). In the same open reading frame preCore is core with a signal peptide at the N-terminus. It undergoes post-translational processing before secretion as hepatitis B e antigen (HBeAg), the function of which remains uncertain, but may be primarily immunomodulatory (M. T. Chen et al. 2004). Three envelope proteins – large, medium and small (L, M and S) are produced from the surface open reading frame and collectively are called HBsAg – S and M in virions and in the various secreted subviral particles, L, which is required for infectivity, predominantly in virions (Gripon et al. 1995). The extra domains in M and L are called PreS2 and PreS1, respectively. X protein, or HBx is a promiscuous transcriptional transactivator that is also required for optimal cccDNA transcription (Gearhart & Bouchard 2010). In addition hepatitis B spliced protein (HBSP) is synthesised from a spliced pregenomic RNA. Its significance is uncertain but it may contribute to fibrosis and to motility of hepatoma cells (Soussan et al. 2003; W. N. Chen et al. 2012). An outline of the HBV replication cycle is depicted in Zoulim & Locarnini 2009 (Zoulim & Locarnini 2009).

Hepatitis B virus infection can give rise to two diseases, acute hepatitis B (less than 6 months from symptom onset to viral clearance) and chronic hepatitis B (CHB) (Lavanchy 2004). CHB is primarily the result of infection in childhood, with high rates of chronic infection after exposure in childhood and up to 90% of infected neonates developing chronic infection (Beasley et al. 1977; Beasley et al. 1982). By early adulthood more than 95% of infections lead to viral clearance, so most epidemiologically important infections occur in childhood (Beasley et al. 1983). There are currently 240 million individuals chronically infected with hepatitis B, which results in around 750,000 deaths annually as a result of cirrhosis or hepatocellular carcinoma (HCC) (Ott et al. 2012; Stanaway et al. 2016). WHO published a strategy to eliminate viral hepatitis in 2016, including targets to treat 80% of eligible individuals with

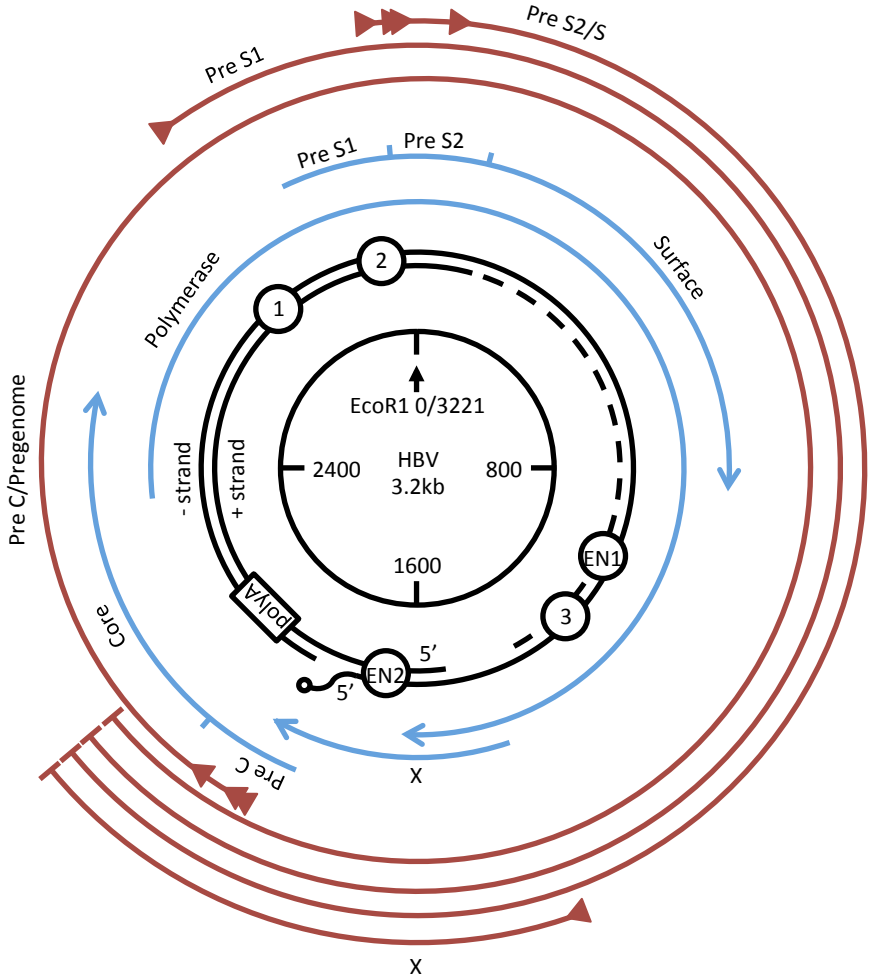

Figure 1.5 Genomic structure of hepatitis B virus.
Adapted from Moolla *et. al.* 2002. Genome coordinates are given relative to the EcoR1 restriction site. The cis-acting regulatory elements are depicted as circles and rectangles. The pregenomic and preS2/S transcripts have heterogenous start sites, but all transcripts share a common 3' end due to the single polyadenylation signal.

hepatitis B and reduce viral hepatitis mortality by 65% by 2030 (World Health Organization 2016). However, even in rich countries it is uncertain whether these goals are achievable (A. Y. Kim 2017).

The natural history of hepatitis B infection is reviewed in Rehermann 2013 and Yim and Lok 2006 (Rehermann 2013; Yim & Lok 2006) (Figure 1.6). Typically, infected children experience 2-3 decades of high viral load without elevation of ALT or the development of liver fibrosis, the so-called “immune tolerant” phase. This period is usually clinically silent, although recent work shows HBV DNA integration and clonal hepatocyte expansion already occurs at this stage, which may be a risk factor for HCC (Mason et al. 2016; Marongiu et al. 2008).This is followed by a period of episodes of inflammation as some degree of immune control is established, known as the immune active phase. Over several years, viral load is reduced and HBeAg seroconversion occurs. Infected individuals can then remain in an HBeAg negative, low HBV viral load (HBV DNA <2000 IU/ml), low-replicative or inactive carrier phase for many years. Progression to liver cirrhosis is associated with high viral load, and this inactive carrier phase carries a relatively good prognosis (Iloeje et al. 2006). There is a roughly 1% rate of HBsAg seroconversion, which is associated with viral control and very good prognosis (Y. C. Chen et al. 2002; Arase et al. 2006). A proportion of patients develop reactivation of their hepatitis, either with HBeAg positive hepatitis, or with HBeAg negative hepatitis as a result of pre-core mutations that reduce or abolish HBeAg production (Liaw & Chu 2009). In European patients, HBeAg negative CHB, characterised by recurrent, spontaneous flares of ALT, is associated with a high risk of progression to cirrhosis and HCC in untreated patients (Bonino et al. 1986). These individuals make up a significant proportion of the burden of CHB in hepatitis clinics of rich countries (Hadziyannis & Vassilopoulos 2001). Hepatitis B vaccine, based on HBsAg expressed in a yeast and administered with alum adjuvant, has been

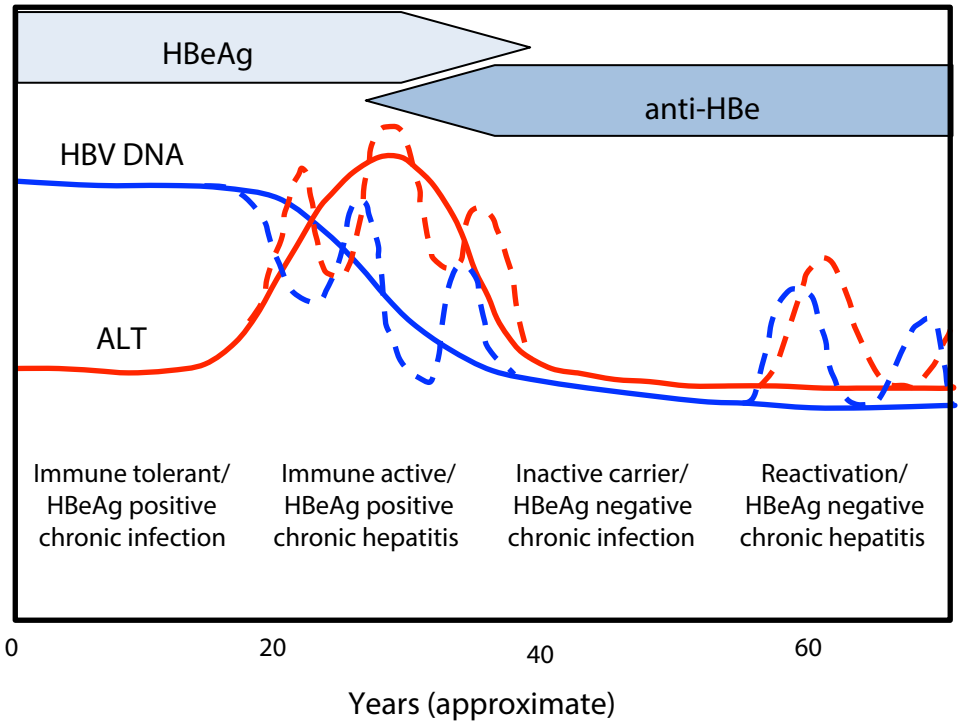

Figure 1.6 Phases of chronic hepatitis B infection
Adapted from Yim and Lok 2006. Typically, chronic hepatitis B infection is acquired in early childhood, and establishes a steady-state with high viral load and low ALT, that may persist for 2-3 decades (HBeAg positive chronic infection). At some point a degree of immune control is established over a period of several years. During this phase HBV DNA and ALT fluctuate due to bouts or flares of hepatitis, and over time the viral load falls and HBeAg, a marker of high level viral replication, is replaced by anti-HBe antibodies (HBeAg positive chronic hepatitis). Following e antigen seroconversion, a state of HBeAg negative chronic infection is established and may persist for many years with low viral load and ALT and little liver damage. Some individuals may suffer reactivation with HBeAg negative chronic hepatitis. Patients may cycle between HBeAg negative chronic infection and chronic hepatitis, with each bout of hepatitis resulting in liver damage that is a risk factor for HCC and contributes to cirrhosis.

know to prevent infection for many years (Whittle et al. 2002). It has been successful in reducing HCC in Taiwan and a 30 year follow-up of vaccinees in the Gambia is due to report soon (M. H. Chang et al. 1997; Viviani et al. 2008). However, hepatitis B transmission continues in settings with limited resources, largely due to the twin difficulties of the need for a first dose on the day of birth in endemic settings, and the risk of transmission from HBeAg positive mothers even if vaccine is administered (Wong et al. 1984; Ekra et al. 2008; C. Q. Pan et al. 2016). Due to the long timecourse of infection and the difficulties in delivering optimal management where it is most needed, hepatitis B-associated mortality is rising, and is expected to continue to rise into the 2040s (Stanaway et al. 2016; Nayagam et al. 2016).

There are two main treatment modalities – pegylated interferon alpha and nucleos(t)ide analogues. Pegylated interferon alpha is administered as injections for a finite duration, has a 20% off-treatment response rate and significant side effects (Sonneveld et al. 2010; Rijckborst et al. 2010). Nucleos(t)ide analogues are well tolerated but do not clear the virus so must be continued indefinitely (Gordon et al. 2013). Viral resistance to these drugs is variable, with over 50% resistance to lamivudine after 5 years of therapy, whereas no tenofovir resistance has been detected after 7 years of treatment (Lok et al. 2003; Buti et al. 2015). Latest guidelines on clinical management, published in March 2017, recommend assessment of the virological and clinical status of the patient to divide patients into four groups: HBeAg positive with chronic infection or chronic hepatitis, HBeAg negative with chronic infection or chronic hepatitis (European Association for the Study of the Liver 2017). These groups replace the old nomenclature of immune tolerant, immune active HBeAg positive, inactive carrier and HBeAg negative chronic hepatitis (European Association for the Study of the Liver 2012). In low-middle income countries, where 80% of the burden of CHB disease occurs, achieving European-level clinical care is often unrealistic (Lozano et al. 2012). There have been efforts to devise guidelines more appropriate for

this setting, but there is little data upon which to base management (Wiersma et al. 2011; World Health Organization 2015). In this and other settings, established guidance on nucleos(t)ide discontinuation would be of benefit programmatically and to individuals; work is ongoing in this area (Papatheodoridis et al. 2016).

1.5 Hepatocellular carcinoma disease and therapy

Hepatocellular carcinoma, primary malignancy of the hepatocytes and the most common primary liver tumour in humans, is the sixth most common human cancer, but the third largest cause of cancer mortality worldwide (Lozano et al. 2012). Half is caused by HBV and 30% by HCV, and as a result it is common in parts of the world where viral hepatitis is prevalent, notably Africa and South/South East Asia (El-Serag 2011). Other major causes include alcohol, non-alcoholic steatohepatitis (NASH), haemochromatosis and alpha-1 antitrypsin deficiency, and aflatoxin may be a cause on its own but is most important as a co-factor in the context of another cause. HCC is the most common cause of cancer death in West African men (Ferlay et al. 2013).

HCC is a heterogeneous disease. Transcriptome clustering studies disagree about whether HBV-associated HCC forms a distinct group, or whether HBV causes HCC in all clusters (Boyault et al. 2007; Hoshida et al. 2009). Pathological diversity may have contributed to the failure of a number of recent trials of systemic therapy in HCC, and it may be that more personalised approaches are required (Llovet & Hernandez-Gea 2014). Around 10% of HCC demonstrate highly lymphocytic infiltrates, and these are associated with improved prognosis (Kawata et al. 1992; Wada et al. 1998). However, data from transgenic mouse models suggests that a cytotoxic T cell infiltrate, driven by the expression of HBsAg by hepatocytes, causes inflammation leading to HCC (Nakamoto et al. 1998; Guidotti & Chisari 2006). This double edged-nature of the lymphocytic response has been highlighted

in more recent work from the same group, showing a role for platelets in the docking and recruitment of CD8 T cells from the liver sinusoids. By this mechanism, antiplatelet therapy reduces inflammation and prevents HCC in HBV-transgenic mice (Sitia et al. 2012; Guidotti et al. 2015). Other cell types are associated with liver inflammation. In a model of acute hepatitis using wild-type splenocytes adoptively transferred into HBV-Env+, RAG-/- mice, NKT cells (NK1.1 intermediate, TCR+) downregulated NKG2D, presumably following contact with RAE-1 expressing HBV-Env+ hepatocytes. Hepatitis could be abrogated by administration of NKG2D blocking antibody, or by depletion of NKG2D positive cells (all NK cells and a proportion of NKT cells) before adoptive transfer). NKG2D blockade also prevented hepatitis and reduced intrahepatic IFNγ and IL4 production after adoptive transfer of splenocytes into 1.3 genome HBV transgenic mice (Vilarinho et al. 2007). In a model of fibrosis and HCC using mice fed on a choline-deficient, high fat diet, a lymphocytic infiltrate of CD4 and CD8 T cells and NKT cells leads to steatohepatitis and cancer. RAG-/- knockout and β2 microglobulin knockout mice were protected from NASH and HCC, whereas Lymphotoxin β receptor 2 and LIGHT knockout mice were protected from HCC but not NASH, and this effect was associated with a reduction of NKT cells infiltrating the liver, suggesting T cells and NKT cells cooperate to induce HCC in a non-redundant manner in this model (Wolf et al. 2014). An alternative model of toxin-induced liver injury comparing fumarylacetoacetate hydrolase (FAH) knockout mice with FAH-/-, RAG-/-, IL-2Rγ-/- mice. FAH-/- mice are unable to metabolise tyrosine, leading to the accumulation of hepatotoxic metabolites. The ensuing hepatitis and fibrosis is lymphocyte-dependent, although these lymphocytes (a mixture of NK, NKT and T cells infiltrate the liver in this model) also maintain survival. Lymphocytes are not required for liver regeneration after hepatitis but are strongly associated with hepatocarcinogenesis, with CD8 T cells and Lymphotoxin β having major roles (Endig et al. 2016).

Various mouse models of HCC have offered instructive insights. Broadly, there are three groups of mouse models of HCC, chemically-induced HCC models, xenograft models and transgenic models (Heindryckx et al. 2009; He et al. 2015). The chemical induction models replicate the cyclical inflammation and fibrosis that causes human HCC, so these models tend to be favoured. Diethylnitrosamine (DEN) is the most widely used model, it is metabolised *in vitro* via cytochrome P450 to form ethyldiazonium ions, which react with nucleophiles including DNA bases. HCC is driven by hepatocyte damage, compensatory proliferation, DNA repair and inflammation. A single administration causes HCC after 50-100 weeks, with a higher rate in males than females (Nakatani et al. 2001). Carbon tetrachloride (CCl_4) causes HCC in a similar timeframe via Kupffer cell activation and ensuing inflammation (Farazi et al. 2006). A choline-deficient diet induces steatosis and subsequent HCC, and has been used as a model of NASH-induced HCC in humans (E. J. Park et al. 2010). Xenograft models involve injecting HCC cell lines either subcutaneously or into the liver in matrigel (orthoptic xenograft). Nude mice are used to prevent rejection, which limits these models to drug susceptibility proof-of-concept studies (Lu et al. 2007). More recently, tumour allograft models have been developed to study immunotherapy, these models are more technically demanding but can shed light on the importance of tumour immune responses, showing for example a synergistic effect of antigen specific CD8 T cells and a tyrosine kinase inhibitor, Sunitinib (Avella et al. 2011). There are multiple transgenic mouse models of HCC. Constitutive expression of HBx, HBsAg and HCV core all induce HCC after 12-18 months (Nakamoto et al. 1998; Lakhtakia et al. 2003; Moriya et al. 1998). Conditional expression of oncogenes such as c-Myc and transforming growth factor α under liver-specific promotors can be used to investigate carcinogenic pathways, but do not induce the inflammation and fibrosis seen in human HCC (Santoni-Rugiu et al. 1996; G.-H. Lee et al. 1992).

HBV is unusual in that it can cause HCC in the absence of cirrhosis, using various mechanisms (Di Bisceglie 2009). By contrast HCC in the context of HCV occurs exclusively in the context of cirrhosis or advanced fibrosis (Lok et al. 2011). DNA integration is common in HBV-associated HCC, and multiple integrations are associated with poor prognosis (Sung et al. 2012). Recent work has demonstrated that DNA integration and hepatocyte expansion are more frequent in the early, "silent" phase of infection than previously recognised, perhaps explaining why HCC risk remains elevated when antivirals are started later in the disease course (Mason et al. 2016). Several authors have reported the integration in the promoter region of the hTERT gene, encoding the enzyme telomerase, which maintains telomere length and is important in stem cells and immortalised malignancy (Gozuacik et al. 2001; Ferber et al. 2003). These integrations lead to over-expression of telomerase, contributing to immortalisation. HBx protein contributes to oncogenesis in a number of ways, including via p53 (S. G. Lee & Rho 2000; Knoll et al. 2011), via interactions with SMAD3 signalling (Murata et al. 2008), and via reactive oxygen species (S. K. Li et al. 2008). HCC in the context of HCV requires a degree of fibrosis/cirrhosis, but also has some direct actions via retinoblastoma protein, oxidative stress and PPARα (Korenaga et al. 2005; Munakata et al. 2007; Tanaka et al. 2008). Inflammation is a significant contributor to HCC pathogenesis (J. Huang et al. 2014; Grivennikov et al. 2010) (Figure 1.7). Viral infection in the liver activates Kupffer cells to produce IL-6 and hepatocyte growth factor, which directly drive hepatocyte proliferation and induce liver cancer in mice (Maeda et al. 2005), and also IL-10, TGFβ and TNFα (Budhu & X. W. Wang 2006). IL-6 in particular is a crucial mediator of HCC and differences in its expression may account for the difference in HCC rates between males and females (Naugler et al. 2007). TNFα induces apoptosis in neighbouring hepatocytes in mice deficient in the tumour suppressor CYLD, which may increase DAMP signalling and encouraging compensatory proliferation (Nikolaou et al. 2012). Both IL-6 and

TNFα are also implicated in obesity-associated HCC in a choline-deficient diet model (E. J. Park et al. 2010). Lymphotoxin α and β are upregulated in HBV, HCV and HCC and their over-expression causes HCC in transgenic mice by inducing chemokines CCL2, CXCL10, CXCL1 and CCL7, which in turn recruit circulating inflammatory cells (Haybaeck et al. 2009). TGFβ promotes proliferation in HBV-infected cells and contributes to retrodifferentiation and epithelial-mesenchymal transition along with IL-6 (Dubois-Pot-Schneider et al. 2014). Not only are multiple pro-inflammatory and carcinogenic pathways activated, but there is also induction of immunosuppressive mechanisms to downregulate anti-tumour surveillance. For example, IL-10 impairs T and NK cell function (Das et al. 2012; Peppa et al. 2010). Recent transcriptomic data has suggested that tumour-infiltrating macrophages in HCC express high levels of IL-10, which may impair the function of NK cells recruited via CXCL10/CXCR3 (Chew et al. 2017).

In rich countries, early stage HCC is curable, but most cases present too late for curative resection. The disease is intrinsically resistant to standard chemotherapy and radiotherapy, as a result average 5-year survival in the USA is less than 15% (El-Serag 2011). Sorafenib, a tyrosine kinase inhibitor, is the only licenced treatment, it improves survival by around three months (Llovet et al. 2008). The prognosis in hepatitis B-associated HCC can be improved by treatment with nucleos(t)ide analogues after tumour resection, although this becomes apparent only after 2 years, so is probably due to control of viral replication and resultant inflammation preventing new primary HCC, rather than by a direct effect (G. Huang et al. 2015). Local therapies such as trans-arterial chemo-embolisation (TACE) or radio-frequency ablation are often used to reduce disease burden, but are not curative (Bruix et al. 2004). As a result of this dire situation, there has been much excitement about the prospect of immunotherapies for HCC (Prieto et al. 2015). Nivolumab, a programmed death 1 (PD-1) blocking antibody was recently

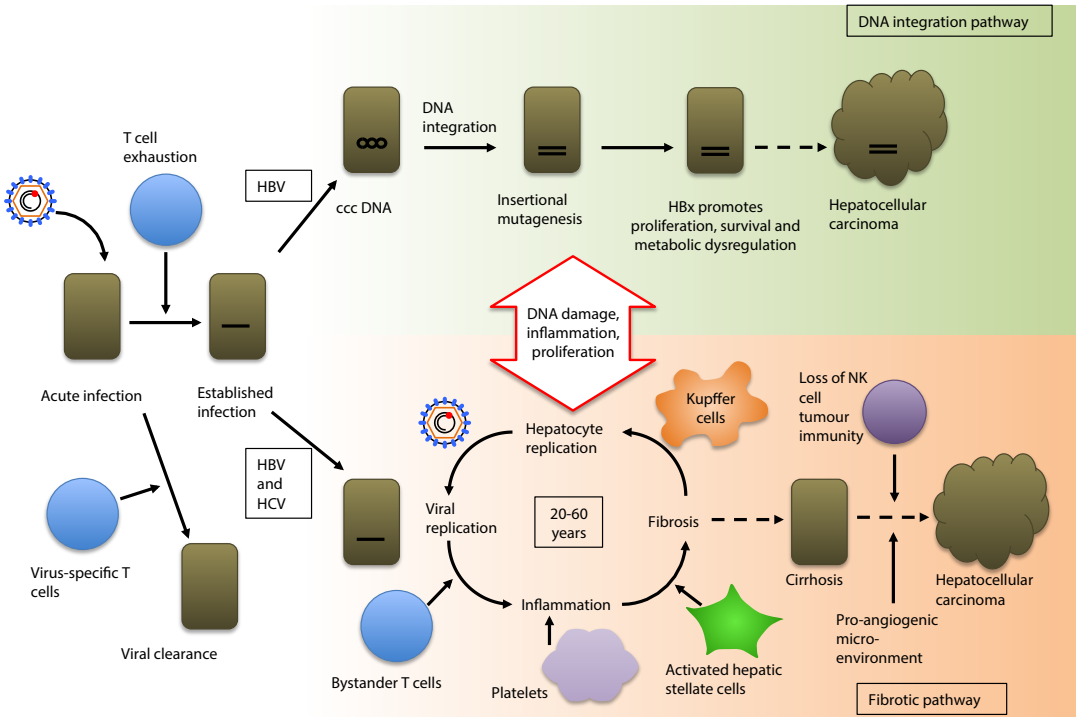

Figure 1.7 Cartoon of mechanisms of HCC carcinogenesis in chronic viral hepatitis
HBV is capable of carcinogenic insults via DNA integration as well as through viral replication and ensuing inflammation. HCV is an RNA virus, so does not interact directly with host DNA, although some HCV proteins have oncogenic function. However the major route of carcinogenesis in both infections is via recurrent inflammation and healing with compensatory clonal proliferation.

shown to have a 20% response rate in HCC in a phase 1-2 trial, phase 3 trials are underway (El-Khoueiry et al. 2017).

There has been some interest in the expression of NKG2D ligands and their potential as immunotherapeutic agents/targets in HCC (Mondelli 2012). In a Japanese cohort, HCC was shown to express MICA and ULBP1, but only ULBP1 was associated with good prognosis (Kamimura et al. 2012). Soluble MICA has been seen in HCC patients from China and Japan, and is associated with impaired peripheral NK cell function and poor prognosis (Jinushi et al. 2003; Kumar et al. 2012; Fang et al. 2014; J.-J. Li et al. 2013). However there are no data on soluble NKG2D ligands in African HCC patients or on the ULBPs. These studies suggest that NKG2D-mediated control of HCC is beneficial. However, experiments with diethylnitrosamine (DEN)-induced HCC in NKG2D knockout mice suggest that NKG2D may drive carcinogenesis by exacerbating inflammation and increasing hepatocyte turnover and compensatory proliferation (Sheppard et al. 2017). Another contradictory finding is that the SNP at rs2596542, associated with MICA shedding in HCC, has differential effects depending on the cause of HCC, with A/A genotype at high risk of HCC in HCV infection, whereas in HBV infection the G/G genotype carries a high risk of HCC (Kumar et al. 2011; Kumar et al. 2012; Tong et al. 2013). Another observation suggesting NKG2D function may be deleterious in HCC is that the high-affinity MICA-129Met allele is associated with increased risk of HCC in the context of HBV (Tong et al. 2013).

1.6 NK cells in the liver and liver diseases

One of the major functions of the liver is as a capillary bed between the gut and the rest of the body, to remove toxic and bacterial products from the portal circulation before they can circulate (Crispe 2009). During normal liver function this must be achieved without inflammation, despite the constant exposure to both foreign antigens and danger signals. As a result, the liver is

a tolerogenic organ, able to be transplanted across HLA haplotypes without requirement for long-term immunosuppression and to protect other co-transplanted organs from rejection (Calne et al. 1969; Rosen 2008). Tolerance is maintained by multiple mechanisms, an important one being hepatic priming leading to T cell deletion (Bowen et al. 2004; Holz et al. 2012). Several infections have evolved to specifically infect the liver and exploit this tolerogenic tendency (Protzer et al. 2012). NK cells, by responding to altered self rather than foreign antigen, can go some way in making up for impaired hepatic T cell immunity without responding to ingested foreign antigen (Rehermann 2015). The fenestra in specialised liver sinusoidal endothelial cells allow intravascular lymphocytes to make direct contact with parenchymal cells to mediate cellular immunity (Guidotti et al. 2015).

NK cells are found in many tissues throughout the body (Björkström et al. 2016). In the liver NK cells comprise 30-50% of the intrahepatic lymphocyte pool (Doherty et al. 1999). They correspond to the pit cells seen in electron micrographs of inflamed liver (Kaneda et al. 1984). They are known to have complex regulatory interactions with Kupffer cells, the resident macrophages of the liver (Tu et al. 2008).

Recent work by our group and others has identified a liver-resident NK cell population characterised by an Eomes-high, Tbet-low transcriptional profile, and by the expression of CD69 and CXCR6 (Stegmann et al. 2016; Harmon et al. 2016; Hudspeth et al. 2016; Lugthart et al. 2016) (Figure 1.8). These cells make up 20-80% of the intrahepatic NK cell population, are a subset of the CD56bright NK cells previously described in the liver, but are distinct from the CD56bright peripheral NK cells. These cells may be long-lived, and IL-15 and TGFβ may be the drivers of the Eomes-high transcriptional profile (Cuff et al. 2016). Liver resident NK cells make similar amounts of IFNγ and have a similar degranulation potential (measured by CD107a expression on

stimulation) to their non-resident but intrahepatic counterparts, however they contain lower levels of granzyme B and perforin *ex vivo* (Stegmann et al. 2016). In addition there is a smaller population (0-12% of the intrahepatic NK cell pool) of CD49a+ NK cells, which are CD56bright but express NKG2C and KIR and have been suggested to be analogous to the CD49a+, DX5- memory NK cells in the mouse (Marquardt et al. 2015; Paust et al. 2010; Peng et al. 2013). They do not express CD57, and contain high levels of granzyme B but low levels of perforin. These cells are Tbet-high, Eomes-low and do not overlap with the CXCR6+ CD69+ liver resident pool.

NK cells are implicated in a variety of liver diseases (Male et al. 2017). Liver fibrosis, driven by activated stellate cells, is a common pathway in inflammatory diseases of the liver. Increased NK cell infiltrate in primary sclerosing cholangitis is associated with reduction in fibrosis, and NK cells have been shown to kill activated hepatic stellate cells in culture via a variety of mechanisms (Berglin et al. 2014; Glassner et al. 2012). *In vivo*, NKp46 has been implicated in killing of hepatic stellate cells and attenuation of liver fibrosis (Gur et al. 2012).

The bulk of NK cell research in human liver disease has focused on viral hepatitis, specifically hepatitis B and C. Immune responses to HBV are reviewed in Bertoletti and Ferrari 2012 and Protzer 2012 (Bertoletti & Ferrari 2012; Protzer et al. 2012). CD8 T cells are crucial for viral clearance, in chronic disease T cells are exhausted due to impaired priming and the tolerogenic nature of the liver (Thimme et al. 2003). It is important to observe that the nomenclature for CHB disease stages is a clinical, not immunological, description. CD8 T cell responses are most easily detectable in patients with “inactive” CHB, and HBV specific T cells are found in “immune tolerant” individuals at similar levels to “immune active” individuals (Maini et al. 2000; Mason et al. 2016). However, there is significant deletion of HBV specific T cells in CHB, particularly where viral replication is high

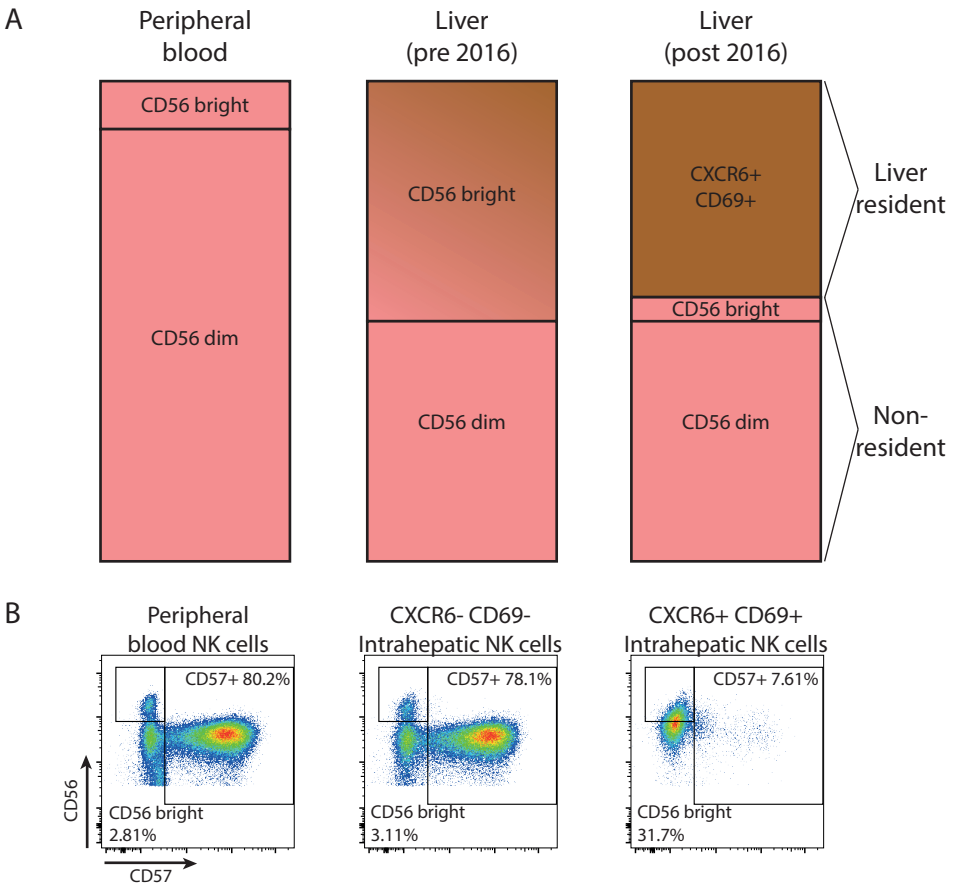

Figure 1.8 Comparison of peripheral blood and intrahepatic NK cell populations.
(A) Peripheral blood NK cells form two populations, the majority CD56dim and a small population of CD56bright NK cells. In the liver there are similar sized populations of CD56 bright and dim NK cells. Following the publication in 2016 of a number of articles it has been shown that the intrahepatic CD56 bright population is comprised primarily of CXCR6+ CD69+, liver resident NK cells with a distinct transcriptional profile.
(B) CXCR6- CD69- intrahepatic NK cells are phenotypically similar to their peripheral blood counterparts. CXCR6+ CD69+ NK cells are phenotypically distinct. They do not express markers associated with highly differentiated NK cells, such as CD57.

(Webster et al. 2004), mediated at least in part by CTLA-4 and BIM (BCL2-interacting mediator of cell death) (Schurich et al. 2011; Lopes et al. 2008). The remaining HBV-specific CD8 T cells are markedly dysfunctional, expressing high levels of the negative regulators PD-1 and Tim-3, inhibiting the production of the important antiviral cytokine IFNγ (Boni et al. 2007; Nebbia et al. 2012). T cell function is further inhibited by metabolic dysfunction induced by myeloid-derived suppressor cells (Pallett et al. 2015). In the relative absence of HBV-specific T cells, non-specific T cells make a significant contribution to liver pathology (Maini et al. 2000; Rehermann 2013), facilitated by neutrophil-derived matrix metallo-proteinases (Sitia et al. 2004).

In NK cells in chronic hepatitis B, there is impairment of cytokine function with preserved cytotoxicity, due to the induction of IL-10 production by HBV (Oliviero et al. 2009; Peppa et al. 2010). NK cells tend to be reduced in number but with a more activated phenotype in the immune active phase compared with in the immune tolerant phase (Sprengers et al. 2006; Tjwa et al. 2011). NK cells can kill infected hepatocytes via TRAIL, but also kill antiviral CD8 T cells via TRAIL, so their overall contribution to pathology may be mixed (Dunn et al. 2007; Peppa et al. 2013; Maini & Peppa 2013) (Figure 1.9). In addition, activated and HBV-specific CD4 T cells in the infected liver upregulate MICA and can be killed by NK cells via NKG2D (W.-C. Huang et al. 2017). Treatment with nucleos(t)ide analogues control viraemia and reduce NK cell activation, with an associated restoration of HBV-specific CD4 T cells (Boni et al. 2015). In contrast, treatment with pegylated IFNα leads to an increase in circulating IL-15, associated with an expansion of activated CD56bright NK cells (Micco et al. 2013; Gill et al. 2016).

In hepatitis C, NK cell cytotoxicity is associated with protection in high-risk individuals, and with better treatment outcomes following pegylated interferon therapy (Khakoo et al. 2004; Golden-Mason et al. 2010; Ahlenstiel et al.

2011; Oliviero et al. 2013). Similar to CHB, IFNγ production is impaired in chronic HCV (Edlich et al. 2011). NKp46 appears to be an important activating receptor associated with control of HCV (Krämer et al. 2012).

In a small series of HCC patients, tumour-infiltrating NK cells had reduced perforin and granzyme B compared with liver NK cells (Cai et al. 2008). This is in keeping with NK cells infiltrating other solid tumours, which are functionally impaired by multiple mechanisms including regulatory T cells, myeloid derived suppressor cells and immunosuppressive cytokines (Guillerey et al. 2016). NK cells are likely to have a role in HCC in the context of HBV, as KIR-HLA interactions predictive of strong NK cell function contribute to early age of onset of HCC (N. Pan et al. 2013). Interestingly, IL-2 stimulated liver-derived NK cells were better than IL-2 stimulated peripheral NK cells at killing HepG2 cells *in vitro* (Ishiyama et al. 2006).

Metastases to the liver are common in a wide range of malignancies, including lung, breast and GI cancers. Colorectal metastases can be studied as they are commonly resected with curative intent (Chua et al. 2012). In a recent study, NK cell infiltration was associated with good prognosis, and has previously been associated with response to chemotherapy (Donadon et al. 2017). This is in contrast to primary colorectal tumours, where few NK cells are found (Halama et al. 2011). Whereas in HCC inflammation may drive carcinogenesis, in colorectal metastases to the liver activation of NLRP3 inflammasome induces IL-18, which drives NK cell mediated anti-tumour responses in mice (Dupaul-Chicoine et al. 2015). In primary colorectal tumours, MICA expression is associated with good prognosis following resection (Watson et al. 2006; McGilvray et al. 2009). This may not be due to an effect in the gut, where NK cells are few and secondary metastases are rare, but may be a consequence of NK cell-mediated clearance of MICA-expressing micrometastases in the liver. However this has not been explored experimentally.

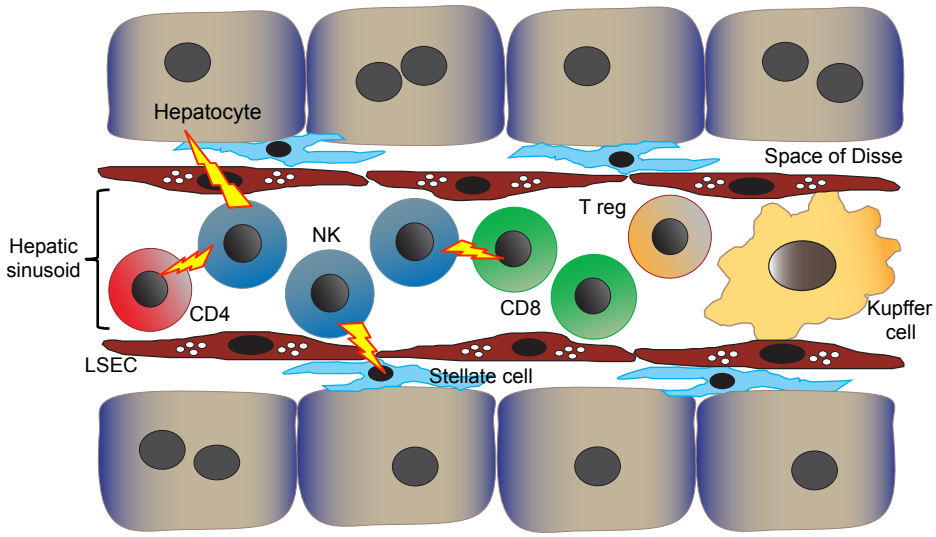

Figure 1.9 NK cell interactions in the liver sinusoid
NK cells have been shown to kill T cells, stellate cells and hepatocytes in various settings including HBV infection. They have regulatory interactions with Kupffer cells and may take advantage of the slow blood flow through the hepatic sinusoid to have interactions with other cell types such as liver sinusoidal endothelial cells (LSEC) or regulatory T cells (T reg). Figure kindly provided by Anna Schurich, Maini group

1.7 NK cell immunotherapy

NK cells are specialised anti-cancer cells, and oncologists have tried to use NK cells to treat human cancers in different ways for many years (Rosenberg et al. 1985), with most success in haematological malignancy.

Various groups have tried to use KIR-ligand mismatched haematopoietic stem cell transfer, and although early results were conflicting (Davies et al. 2002; Ruggeri et al. 2007), with improved patient selection and concentrating on the most promising clinical scenarios there may be a role for KIR-ligand mismatch in acute myeloid leukaemia (Heidenreich & Kroger 2017) and clinical trials are ongoing (NCT02646839). Activating KIR may also have an important role to play (Mancusi et al. 2015; Stringaris et al. 2010).

In solid tumours, progress on NK cell immunotherapy for cancer has been slower (Morvan & Lanier 2016). One of the problems is the state of anergy induced in NK cells by the tumour environment (Ardolino et al. 2014). Checkpoint blockade targeting T cells has been a significant advance in cancer immunotherapy (Couzin-Frankel 2013). It may be that similar approaches could be used in NK cells, however PD-1 expression by NK cells is absent in most people, and where present seems to be driven by CMV rather than tumour (Pesce et al. 2017; Chiesa et al. 2016). However NK cells can express LAG3 and Tim3, and KIR blockade might be analogous to checkpoint inhibition for NK cells - anti-KIR antibodies are safe in humans (Vey et al. 2012).

NKG2D ligands make attractive targets for cancer immunotherapy, as they are upregulated by a wide variety of tumours (Spear et al. 2013). One logical approach is to use the binding domain in NKG2D but with one or more signalling domains to give strong activating signals, an NKG2D chimeric antigen receptor (NKG2D-CAR) and transfect this receptor into a cytotoxic T or NK cell for adoptive transfer (Sentman & Meehan 2014). Transfection of

NK cells with a NKG2D-DAP10-CD3ζ CAR improved NK cell cytotoxicity and control of a mouse model of osteosarcoma (Y.-H. Chang et al. 2013).

ADCC is another important mechanism by which NK cells can control tumours (W. Wang et al. 2015). Rituximab was the first tumour antibody shown to act via ADCC in control of B cell lymphomas (Cartron et al. 2002; Dall'Ozzo et al. 2004). In solid tumours, Trastuzumab and Cetuximab, for HER2+ breast cancer and EGFR+ colorectal cancer respectively, also achieve at least some of their activity via ADCC. As techniques in molecular biology progress, novel bispecific proteins combining antibody variable regions, often smaller than full Fab fragments, and NKG2D ligands, are being developed to recruit NK cell activity against tumours such as colorectal cancer and HCC (Rothe et al. 2013; T. Wang et al. 2016).

The use of cytokines as therapeutic agents for cancer has been limited by their toxicity (Floros & Tarhini 2015). However IL-15 has been shown in a number of settings to enhance the anti-tumour properties of NK cells (Sutherland 2006; Mao et al. 2016). As a result researchers have sought ways to utilise the IL-15 effect while avoiding the toxicity associated with systemic administration. IL-15 signalling drives persistence in IL-15-CAR T cells (Hurton et al. 2016), and a similar approach has been tested in NK cells. An NK cell line transfected with IL-15 signals in an autocrine fashion to control HCC in mouse models (Jiang et al. 2014). The group of Dario Campana has used transfection with a membrane bound IL-15 (mbIL-15) constructs in different ways to augment NK cell anti-cancer function. K562 cells transfected with mbIL-15 and 4-1BB ligand can expand and activate NK cells for use in HCC immunotherapy (Kamiya et al. 2016). Retroviral transduction of human NK cells with mbIL-15 enhanced persistence, cytotoxicity and tumour control in a model of human AML in NOD/scid/IL2R gamma null mice (Imamura et al. 2014). An IL-15 superagonist ALT-803, that combines IL-15, IL-15Rα and an Fc domain that improves the half life without

recruiting complement or ADCC, has been investigated as an anti-cancer agent, but in humans may have similar toxicity to IL-15 alone (Rosario et al. 2016). One way to reduce toxicity might be to target IL-15 to the tumour environment, as has been demonstrated *in vitro* with an ALT-803-rituximab fusion protein (B. Liu et al. 2016).

1.8 Aims of this thesis

1. To comprehensively investigate the NKG2D receptor-ligand system in the setting of hepatocellular carcinoma by assessing soluble NKG2D ligands and their effect on NKG2D expression and function in circulating and tumour-infiltrating NK cells.

2. To construct a model of the interaction between tumour-infiltrating NK cells and HCC to be used to interrogate the functional consequences of NKG2D/NKG2D ligand interactions in this setting.

1.9 Knowledge Gaps

The release of soluble NKG2D ligands other than MICA/MICB by HCC and many other solid tumours is not known.

Although the expression of soluble and cell surface NKG2D ligands by HCC has been associated with poor and improved prognosis respectively, this data is purely observational. No investigations of the effect of NKG2D expression on NK function in this setting exist.

There is uncertainty about the functional consequences of the presence of soluble NKG2D ligands on NK cells in humans, particularly whether soluble NKG2D ligands can protect against NKG2D downregulation and enhance cytotoxicity.

NK cells are known to infiltrate HCC, but it is not known whether these NK cells infiltrate from the surrounding liver tissue or the bloodstream, what mechanisms inhibit their function or whether function might be restored by targeting the NKG2D pathway.

The contribution of NK cells to the infiltrating lymphocyte pool in metastases from non-HCC primary tumours eg colonic adenocarcinoma to the liver is unknown.

NK cells are known to lyse tumour cells, but NK cells infiltrating solid tumours lose activity via unknown an mechanism(s). A model of NK cells infiltrating a solid tumour could be used to investigate agents to impair or reverse this mechanism.

1.10 Hypothesis

NK cells, via NKG2D-mediated interactions, can successfully control HCC through cytotoxicity or other effector functions, but HCC is able to evade this control by manipulation of NKG2D ligands, thereby manipulating NKG2D function in NK cells, and that it is possible to intervene to restore NKG2D function in tumour-infiltrating NK cells.

1.11 Research Questions

1. Which soluble NKG2D ligands are released by hepatitis B-associated HCC?

2. Does soluble ligand release affect NKG2D expression by circulating NK cells and if so, does this impact effector function?

3. Does NKG2D ligand expression have prognostic association and are these independent of disease staging and other known prognostic markers?

4. Is NKG2D ligand expression related to the aetiology of the HCC?

5. Is soluble NKG2D ligand expression seen in secondary liver tumours?

6. What is the mechanism by which NKG2D ligands are induced?

7. Do NK cells infiltrating HCC resemble circulating or intrahepatic NK cells?

8. Do tumour-infiltrating NK cells have altered NKG2D expression?

9. Do tumour-infiltrating NK cells have altered effector function?

10. Does exposure to HCC in vitro alter NK cell NKG2D expression or effector function?

11. Can NK cell effector function be protected from the effect of HCC by targeting the NKG2D pathway?

12. Can other pathways be used to maintain NK cell effector function in the context of HCC?

Chapter 2 Materials and Methods

2.1 NKG2D Ligand ELISA

ELISAs for MICA, MICB, ULBP1, ULBP2 and ULBP3 (R&D) were performed as per the manufacturer's instructions. Briefly, 96 well flat bottom plates (Falcon) were coated overnight at room temperature with capture antibody diluted in phosphate buffered saline (PBS) (Sigma). Plates were washed three times by mechanical plate washer in wash buffer comprised of PBS with 0.05% Tween-20 (Fisher), and blocked for one hour in a reagent diluent of PBS with 1% fraction V BSA (Fisher). Thawed patient serum was diluted one part in three with reagent diluent and standards and a blank were prepared by serial dilution in the same reagent diluent. All samples and standards were assayed in duplicate. Samples were incubated for two hours at room temperature and the plates were washed three times. Detection antibody diluted in reagent diluent was added at the recommended concentration and incubated for a further two hours before washing three times. Streptavidin HRP at 1:200 in reagent diluent was added for 20 minutes and a final three washes performed. The plates were developed using Substrate Reagents A+B (R&D) for 20 minutes in the dark and the reaction stopped with Stop Solution (2N HCl) (R&D). Plates were read immediately using an automated plate reader (Finstruments Multiskan) at 450nm. Mean optical densities (OD) for each standard and sample were taken and antigen concentrations were calculated using Excel for Mac 2011 (Microsoft) to calculate a trend line of the format $y=ax^b$ in order to interpolate a concentration from the Mean OD. Statistical tests were performed using Graphpad Prism 6.

2.2 Exosome isolation

Exosomes were isolated from serum using Izon Science qEV Size Exclusion Columns according to the manufacturer's instructions.

2.3 Tumour-conditioned media

Small fragments of tumour (less than 0.1g) were weighed. Their mass in mg was multiplied by 10 and this many microlitres of CRPMI+10% FBS was added. The tissue was incubated overnight at 37°C. The supernatant was removed and centrifuged in a microcentrifuge at 2000 rpm for 5 minutes to pellet any cells or debris. The supernatant was removed for use in ELISA.

2.4 Imagestream

For identification of exosomes in serum, Fc receptor blocking reagent (Miltenyi) was added to paired serum, exosome and protein fractions before staining with BODIPY-FL (ThermoFisher), CD45 PE-Cy7 (eBioscience) and ULBP1-PE (R&D). Samples were fixed with Cytofix (BD) before acquisition. For NKG2D internalisation assays NK cells were surface stained with CD56 PE-Cy7 (Beckman Coulter), CD3 PE Texas Red (BD) and 1:10,000 DAPI (ThermoFisher) and fixed in cytofix/cytoperm before intracellular staining with NKG2D Alexa 488 (R&D). Samples were acquired by ImagestreamXL (Amnis) and data analysis performed using IDEAS software (Amnis).

2.5 PBMC isolation and storage

PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare). Peripheral blood was collected into vacutainers containing EDTA (BD) or lithium heparin (BD). Using 15ml falcon tubes (Sarstedt), 10ml blood was carefully layered onto 5ml Ficoll-Hypaque and centrifuged at 2000 rpm, acceleration 6, brake 4 in a Thermo Multifuge centrifuge. The PBMC layer was removed using a Pasteur pipette and washed in Roswell Park Memorial Institute (RPMI) medium (Gibco). Cells were either stained immediately for flow cytometry or counted using trypan blue (Sigma) and transferred to freezing medium (FBS (Sigma) with 10% dimethylsulfoxide (DMSO) (Sigma) at a concentration of 10 million cells/ml for cryopreservation, initially at -80°C before transfer to liquid nitrogen for long-

term storage. Before use cells were thawed in a water bath at 37°C and washed in 20ml RPMI.

2.6 Intrahepatic lymphocyte isolation from biopsies

Liver biopsies were separated into single cell suspension by mechanical dissociation by hand. A cell scraper (Sarstedt) was used to gently disperse the liver cells into RPMI. Clumps of cells were removed by filtration with 70μm filter (Greiner), cells were washed in RPMI and used immediately.

2.7 Intrahepatic lymphocyte isolation from tissue explants

Single cell suspensions from surgical resection of liver and tumour tissues were generated by enzymatic digestion and mechanical dissociation as described in(Stegmann et al. 2016). In brief, tissues were cut into small pieces and incubated for 30 minutes at 37°C in HBSS buffer containing 0.0001% Danas (Roche) and 0.01% collagenase (ThermoFisher). Samples were transferred to C-tubes (Miltenyi Biotec) and processed by gentleMACS (Miltenyi Biotec) using the liver program. Tissue and supernatants were filtered by 70μm filter and centrifuged at 500rpm to knock down large hepatocyte clumps. The supernatant was centrifuged and the pellet resuspended in 30% percoll (GE Healthcare) before further centrifugation at 2000rpm for 10 minutes. The pellet was resuspended in HBSS and layered onto Ficoll-Hypaque as for peripheral blood separation. The lymphocyte layer was removed and cells counted by ADAM counter (NanoEntek) and used immediately.

2.8 Intrahepatic lymphocyte isolation from liver perfusates

Organ transport and perfusion fluid from liver transplantation may contain large numbers of intrahepatic lymphocytes. Perfusion fluid is centrifuged in 50ml falcon tubes (Sarstedt) at 1800 rpm for 15 minutes and the supernatant discarded. The tubes are then vortexed to disrupt the pellet and the cells

pooled and resuspended in RPMI before density gradient centrifugation over Ficoll Hypaque as above.

2.9 Flow cytometric staining

All samples were treated with Fc receptor blocking reagent (Miltenyi Biotec) before staining. Surface staining was performed in 96 well plates (Sarstedt) in staining buffer of 50% PBS, 50% Brilliant Violet staining buffer (BD). Fixable live/dead stain (Life Technologies) was added to the staining buffer. Antibody staining was conducted for 15 minutes at 37°C in the dark before washing with PBS. Samples for surface staining only were fixed in Cytofix (BD). Samples for intracellular staining were fixed in cytofix/cytoperm (BD) for 20 minutes at 4°C in the dark before staining with intracellular antibodies in saponin buffer (PBS + 1% FBS (Sigma) + 0.1% saponin (Sigma)) for 30 minutes at 4°C in the dark. Samples were then washed once in saponin buffer and once in PBS.

Intranuclear staining was performed using FoxP3 staining buffer (BD). Surface staining was as above, then cells were fixed in buffer A for 10 mins at room temperature followed by buffer A with 1:50 buffer B for 30 mins at room temperature. Samples were washed in PBS and intranuclear staining performed in PBS for 30 mins at 4°C in the dark.

For staining for phospho-ERK, stimulated cells were immediately cooled on ice to minimise alterations in pERK, then conjugated antibodies for surface markers were applied on ice for 30 mins. Cells were washed in PBS, then fixed in Cytofix for 10 mins at 37°C. Permeablisation in Perm III buffer (BD) for 25 mins at -20°C was followed by staining with anti-pERK (Cell Signalling Technologies) for 30 mins at 4°C.

Single fluorochrome compensation controls were made using compensation beads (BD). Compensation matrices were calculated initially in FACSDiva

and edited where necessary in FlowJo X (TreeStar). Samples were acquired on LSR Fortessa or Fortessa X20 (BD) and data analysed in FlowJo X.

2.10 Image Analysis and Quantitation of NKG2D internalisation

Magnetic bead isolated NK cells were cocultured with PLC/PRF/5 cells as described below. Cells were surface stained with anti CD3 PE-CF594, anti CD56 PE-Cy7, permeablised and stained intracellularly with anti NKG2D APC and then DAPI nuclear stain. Events were acquired on the Imagestream^X (Amnis) and data transferred to IDEAS software (Amnis) for analysis. Single cell events were identified by gating on Brightfield Area vs Brightfield Aspect Ratio. A histogram of Brightfield Gradient Root Mean Square was used to identify events in focus. CD56 positive, CD3 negative events within this population were gated on. Using the built-in “Internalization Wizard” an object mask was drawn around the cells using the brightfield image, and eroded 4 pixels. For each cell a ratio of the logarithm of the intensity of the internal brightness to the total brightness was generated, using the APC channel. The ratio was scaled by the wizard such that cells with half the signal intensity internally (50:50 surface:internal brightness) had an internalisation value of 0, and cells with positive values had predominantly internal NKG2D staining.

2.11 Media

Lymphocyte short-term cultures were in complete RPMI + 10% FBS. To 500ml RPMI was added: 10ml 1M Hepes, 10ml 50x MEM essential amino acids, 5ml 100x MEM non-essential amino acids, 0.5ml 50mM 2-mercaptoethanol, 2.5ml 100mM sodium pyruvate, 5ml penicillin/streptomycin 10,000IU/ml (all Gibco), 50ml FBS.

PLC/PRF/5 cells, HepG2 cells and K562 cells were grown in complete RPMI +8% FBS, prepared as above but with 40ml FBS and 5ml amphotericin B

250μg/ml (Sigma). Cell lines were cultured in 75cm^2 or 175cm^2 flasks (Nunc) and split using trypsin-EDTA (Gibco).

HepG2.117, HepG2 AD38 and HepG2 NTCP cells were grown in a containment level 3 laboratory in Dulbecco's modified Eagle medium (DMEM) (Gibco) + 10% FBS + 5ml penicillin/streptomycin.

2.12 NK cell isolation

Untouched NK cells were isolated from PBMC or intrahepatic lymphocytes using magnetic beads by negative selection, according to the manufacturer's instructions (Miltenyi Biotec), achieving >95% purity and viability.

2.13 NK cell – PLC/PRF/5 cocultures

PLC/PRF/5 cells were plated at a density of 50,000 cells/well in 48 well plates (Costar) and incubated in 0.5ml CRPMI+8% FBS for three days to adhere to the bottom of the well and grow to semi-confluence. Media was changed and isolated NK cells were added at 200,000 per well, centrifuged at 300 rpm for 3 minutes and incubated at 37°C for 12 hours. Reagents added the appropriate conditions were: recombinant human NKG2D final concentration 1μg/ml (Sino Biological), anti-MICA (R&D), recombinant human MICA final concentration 1μg/ml (Sino Biological), recombinant human ULBP1 final concentration 1μg/ml (Sino Biological), anti-DNAM1 final concentration 4μg/ml (R&D), anti-TGFβ receptor final concentration 2μg/ml (R&D), anti-IL6 receptor final concentration 4μg/ml (R&D), anti-HLA ABC (BioLegend), recombinant human IL-15 final concentration 1ng/ml (R&D). 0.4μm transwell inserts (Falcon) and 3-day PLC/PRF/5 media mixed 1:1 with fresh CRPMI+10% FBS were used for contact experiments. Supernatants containing NK cells were removed and washed with PBS containing EDTA (Sigma) and FBS. For some experiments, removed NK cells were resuspended in fresh medium and incubated for 18 hours in the presence of

IL-2 20IU/ml (Miltenyi biotec), IL-12 12.5pg/ml (R&D), IL-18 5ng/ml (MBL), IL-15 1ng/ml (R&D), IFNα 10U/ml (PBL Interferon) or medium alone.

2.14 Primary Human Hepatocyte culture and HBV infection

These experiments were performed by Dr Ana Maria Ortega Prieto and colleagues in the laboratory of Dr Marcus Dorner at Imperial College London. Their methodology has recently been accepted for publication as: Ortega-Prieto AM, Skelton JK, Wai SN, et al (2018). 3D microfluidic liver cultures as physiological preclinical tool for hepatitis B virus infection, Nature Communications, In Press.

Cells – Cryopreserved PHH (PHH) were provided by QPS laboratories (Newark, NJ, USA) and Life Technologies (Carlsbad, CA, USA). Briefly, PHH were resuspended in prewarmed thawing medium (QPS laboratories). PHH were centrifuged at 100 × g for 10 min, and resuspended in Williams E medium (WEM) supplemented with Thawing/Plating Supplement Pack (Invitrogen, Paisley, UK) that consisted in 5% Fetal Bovine Serum (FBS), 1 µM Dexamethasone, 100 IU/mL Penicillin, 100 µg/mL Streptomycin, 4 µg/ml Human Recombinant Insulin, 2 mM GlutaMAX^{TM} and 15 mM HEPES, pH 7.4. Cell viability was determined using trypan blue (Sigma-Aldrich, Dorset, UK) and was above 90% in all cases.

Microfluidic 3D culture system – Cells were seeded into isolated bioreactors within the LiverChip platform (CNBio Innovations, Welwyn Garden City, Hertfordshire, UK) with flow in the downward direction on top of collagen-coated scaffolds for 8 hours at a flow rate of 1.0 µl/s. Following cell attachment within the scaffold, the flow was changed to the upward direction and maintained at 1.0 µl/s for the remainder of the culture. Hepatocyte monocultures were seeded at a density of $0.6x10^6$ viable cells in 1.6 ml of medium per well. The cells were maintained in WEM supplemented with Thawing/Plating Supplement Pack (Invitrogen) for the first 24 hours of culture

and in WEM supplemented with Cell Maintenance Supplement Pack (Invitrogen) thereafter for the duration of the culture. The media was replaced every 48h.

HBV patient-derived viruses and infections – HBV positive serum samples of four different donors were used at m.o.i. of 100 genome equivalents per cell. For the 3D infections, serum samples were diluted in 1.6 mL of WEM supplemented with Cell Maintenance Supplement Pack with dexamethasone or hydrocortisone (Invitrogen). HBV infection was performed 3 days after seeding for 24 hours, after which cells were washed three times with maintenance medium for 3.5 min using 1 μL/s downward flow. 2D infections were performed using PHH seeded in 24 well collagen-treated plates. Infections were carried out for 24 hours, after which cells were washed three times and medium was replaced with maintenance medium.

2.15 HCC tissue slices

For some experiments, ex vivo HCC tissue was used instead of cell lines. Cut pieces of HCC tissue were provided by the Tissue Access for Patient Benefit service (TAPb) at the Royal Free Hospital. A 5mm punch biopsy (Stiefel) was used to prepare cores or uniform diameter, from which 1mm slices were cut. Thin 5mm circles of HCC tissue were carefully placed in wells of round bottom 96 well microtitre plates and allowed to rest for four hours in CRPMI+8% FBS. Magnetic bead isolated NK cells were stained with Cell Trace Violet (Life Technologies) as per the manufacturer's protocol before addition to the HCC slices as above.

2.16 CD107a assays

NK cells were mixed with K562 cells at 1:1 effector:target ratio in 200μl CRPMI+10% FBS in 96 well, round bottom plates (Sarstedt). Brefeldin to a final concentration of 1μg/ml (Sigma) and anti-CD107a V450 were added and

the plate centrifuged at 300rpm for 3 minutes before incubation at 37°C for three hours before staining for flow cytometry as above.

2.17 Cytokine stimulation

PBMC, intrahepatic and intra-tumour lymphocytes were stimulated with 5ng/ml IL-12 and 50ng/ml IL-18, or 1ng/ml IL-15 for 12 hours in the presence of 1μg/ml brefeldin, before intracellular cytokine staining as above.

2.18 Immunohistochemistry

Formalin fixed, paraffin-embedded sections of paired tumour and liver tissue were dewaxed and rehydrated, and antigen retrieval performed as described previously (Dunn et al 2007). Endogenous peroxidase activity was quenched with 10% hydrogen peroxide (VWR) in PBS before blocking with 2.5% horse serum (Vector Labs). Anti-ULBP1 antibody (Santa Cruz Biotech) was applied at 1:200 in PBS with 1% BSA (Sigma) overnight at 4°C. Slides were washed and primary antibody detected with ImmPress Horseradish Peroxidase Polymer Detection Kit for 30 minutes. After further washes antibody was visualised by Impact DAB (Vector Labs), then counterstained with haematoxylin (Sigma) and dehydrated before mounting.

2.19 Statistical analysis

The Mann Whitney-U test was used for comparisons of two unpaired groups where n=6 or greater, unpaired t test with Welch's correction was used for comparisons of smaller groups. The Wilcoxon signed rank test was used for comparisons of two paired groups. Spearman rank test was used for correlations of continuous variables. These tests were performed in GraphPad Prism version 6.

Multiple linear regression and Poisson regression were performed in STATA (Statacorp) version 13. MANOVA was performed in SPSS (IBM) version 24.

P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001

2.20 Research Ethics Approval

Blood and tissue sampling in Gambian patients was approved by the Scientific Coordinating Committee of the MRC Unit, The Gambia as SCC1379 and SCC1266.

Blood and tissue sampling of UK patients was approved by the University College London-Royal Free Hospital Research Ethics Committee, ref nos. 11/WA/0077 (liver explants), 11/H0720/4 (liver perfusates), 10/H0720/34 (HCC serum cohort). Blood sampling from healthy donors was approved by the South East Coast Research Ethics Committee, ref no. 11/LO/0421.

Table 2.1 List of reagents

Reagent	Cat No.	Manufacturer
MICA DuoSet ELISA kit	DY1300	R&D
MICB DuoSet ELISA kit	DY1599	R&D
ULBP1 DuoSet ELISA kit	DY1380	R&D
ULBP2 DuoSet ELISA kit	DY1298	R&D
ULBP3 DuoSet ELISA kit	DY1517	R&D
Phosphate buffered saline	P4417-100TAB	Sigma
Tween-20	BP337-500	Fisher
Fraction V BSA	BP1605-100	Fisher
Substrate reagents A+B	DY999	R&D
Stop Solution	DY994	R&D
Fc receptor blocking	130-059-901	Miltenyi Biotec
BODIPY-FL	B10250	ThermoFisher
Cytofix	554655	BD
Cytofix/cytoperm	554722	BD
Phosflow Perm Buffer III	558050	BD
DAPI	D1306	ThermoFisher
Ficoll-Hypaque	17-1440-02	GE Healthcare
RPMI	11879-020	Gibco
DNAase	11 284 932 001	Roche
Collagenase	17104-019	ThermoFisher
Percoll	17-0891-01	GE Healthcare
Brilliant Stain buffer	566349	BD
Trypan Blue	T8154-100ML	Sigma

Fixable live/dead aqua	L34957	Life Technologies
Fixable live/dead blue	L23105	Life Technologies
Saponin	S-7900	Sigma
Fetal bovine serum	F9665-500ML	Sigma
DMSO	D2650-100ML	Sigma
FoxP3 staining buffer	560098	BD
CompBeads	51-90-9001229	BD
Hepes	15630-056	Gibco
MEM Essential amino acids	11130-036	Gibco
MEM Non-essential amino acids	11140-035	Gibco
Mercaptoethanol	31350-010	Gibco
Sodium pyruvate	11360-039	Gibco
Penicillin/Streptomycin	15140122	Gibco
Amphotericin B	A2942-100ML	Sigma
Trypsin-EDTA	25300-062	Gibco
DMEM	61965-026	Gibco
NK cell isolation kit	130-092-657	Miltenyi Biotec
Recombinant human NKG2D	10575-H07B	Sino Biological
Anti-MICA	MAB13001-100	R&D
Recombinant human MICA	12302-H08H-25	Sino Biological
Recombinant human ULBP1	10679-H08H-25	Sino Biological
Anti-DNAM1	MAB666-100	R&D
Anti-TGFβ receptor	AF-241-NA	R&D
Anti-IL6 receptor α	MAB277-100	R&D
Anti-HLA ABC	311412	BioLegend

Recombinant human IL-15	247-IL	R&D
Recombinant human IL-2	130-093-903	Miltenyi
Recombinant human IL-12	219-IL	R&D
Recombinant human IL-18	B001-5	MBL
Recombinant human IFNα	11100-1	PBL Interferon
EDTA	E7889-100ML	Sigma
Brefeldin	B6542	Sigma
Hydrogen peroxide	101284N	VWR
2.5% Horse Serum	MP-7402	Vector Labs
Anti-ULBP1	sc-53131	Santa Cruz Biotech
HRP Polymer Detection Kit	MP-7402	Vector Labs
Impact DAB	SK-4105	Vector Labs
Haematoxylin	GHS332-1L	Sigma
Cell Trace Violet	C34557	Life Technologies

Table 2.2 Antibodies for flow cytometry

Target	Clone	Cat No.	Manufacturer
CD45 BUV805	HI30	564914	BD
CD45 PE Cy7	HI30	25-0459-42	eBioscience
CD56 PE-Cy7	NCAM16.2	335826	BD
CD56 ECD	N901	A82943	Beckman Coulter
CD3 BV711	OKT3	317328	BioLegend
CD3 PE-Cy7	UCHT1	25-0038-42	eBioscience
CD3 BUV395	UCHT1	563546	BD
CD3 PE-CF594	UCHT1	562280	BD
CD16 APC-Cy7	3G8	557758	BD
NKG2D Alexa 488	149810	FAB139G	R&D
NKG2D APC	1D11	320808	BioLegend
NKG2C APC	134591	FAB138A	R&D
NKG2A Alexa 700	131411	FAB1059N	R&D
NKp46 BV605	9E2	331925	BioLegend
NKp30 PE	AF29-4D12	130-092-483	Miltenyi Biotec
CXCR6 PE	K041E5	356004	BioLegend
CXCR6 BV421	K041E5	356014	BioLegend
CXCR6 PE Dazzle	K041E5	356016	BioLegend
CXCR6 APC	K041E5	356006	BioLegend
HLA-DR e450	L243	48-9952-42	eBioscience
HLA-DR V500	G46-6	561224	BD
CD69 PE Dazzle	FN50	310942	BioLegend
CD69 BV605	FN50	310938	BioLegend

CD69 BV785	FN50	310932	BioLegend
IFNγ BV605	4S.B3	502535	BioLegend
IFNγ FITC	25723	IC285F	R&D
CD107a V450	H4A3	560647	BD
CD57 e450	TB01	48-0577	eBioscience
CD57 FITC	TB01	11-0577-42	eBioscience
CD8 Alexa 700	OKT8	56-0086-82	eBioscience
CD8 BV785	RPA-T8	301045	BioLegend
Perforin PerCP Cy5.5	dG9	308113	BioLegend
Perforin APC Cy7	dG9	308128	BioLegend
CD25 APC-eFluor780	BC96	47-0259-42	eBioscience
Granzyme B Alexa 700	GB11	560213	BD
ULBP1 PE	170818	FAB1380P	R&D
ULBP2/5/6 APC	165903	FAB1298A	R&D
ULBP3 PE	166510	FAB1517P	R&D
MICA/B PE	6D4	558352	BD
PVR PE Cy7	SKII.4	337614	BioLegend
Nectin-2 APC	TX31	337412	BioLegend
HLA-ABC PE Cy7	W6/32	25-9983-42	eBioscience
HLA-E APC	3D12HLA-E	17-9953-42	eBioscience
Ki67 FITC	B56	556026	BD
Caspase 3 PE	C92-605	550821	BD
MCL-1 PE	D2W9E	65617S	Cell Signalling
pERK (T202/Y204) APC	197G	13148S	Cell Signalling

Chapter 3 Serum NKG2D ligands in HCC and CHB

3.1 Introduction

The Prevention of Liver Fibrosis and Cancer in Africa (Prolifica) study, led by Prof Mark Thursz at Imperial College London and funded by the EU, consists of two clinical cohorts and a platform for laboratory investigation of liver disease in West Africa. The first clinical cohort, the West African Treatment Cohort for Hepatitis B (WATCH), consists of patients screened for HBsAg by point of care test in the Western region of The Gambia (Lemoine et al. 2016). The study team visited 54 randomly chosen census enumeration areas, 27 rural and 27 urban, and individuals over 30 years of age were offered HBsAg testing (the national hepatitis B vaccination programme commenced in 1990). Individuals who tested positive were offered full clinical assessment (clinical examination, liver function tests, liver ultrasound, fibroscan and HBV DNA viral load measurement) at the MRC Fajara liver clinic. In addition, some HBsAg negative participants received clinical assessment in order to establish normal ranges and to act as controls. A number of additional patients were added following HBsAg testing before blood donation at Edward Francis Small Teaching Hospital, as well as a small number of cases in Keneba and Manduar, the villages where the first hepatitis B vaccination studies were performed (Whittle et al. 2002). Treatment of cases here as soon as HBV treatment was offered in The Gambia was a condition of the original studies. Patients were treated with tenofovir according to EASL 2012 guidelines (European Association for the Study of the Liver 2012). HBeAg was not routinely performed as this is not required to make treatment decisions.

The second clinical study, the Hepatocellular Carcinoma Case-Control study (HC4), involved clinical assessment (as for WATCH) of patients referred to the Fajara liver clinic with suspected liver disease or HCC. Individuals were

referred from a number of surrounding medical facilities with symptoms including jaundice, ascites, abdominal mass. Patients were also recruited to HC4 in Senegal and Nigeria, but all the data in this work relates to patients recruited at MRC Unit The Gambia, Fajara. Patients were recruited before clinical assessment, so this cohort contained patients with HCC and with a range of other diagnoses (liver cirrhosis, active hepatitis B, other abdominal malignancy or liver mass, congestive cardiac failure) in order to establish risk factors for HCC in West Africa.

Diagnosis of HCC was based on histology in some cases, and in others a combination of liver mass on ultrasound, relevant risk factor for HCC and positive alphafetoprotein (AFP). Where biopsy was not performed, it is difficult to distinguish between AFP-negative HCC and a liver mass with an alternate diagnosis as ultrasound has a poor specificity. Unfortunately, AFP has a poor sensitivity, so there will have been a number of true HCCs in this group (T.-S. Chang et al. 2015).

It is known that NK cells can have multiple roles in CHB and cancer (Maini & Peppa 2013; Morvan & Lanier 2016). In Asia, the soluble form of the NKG2D ligand MICA has been associated with improved prognosis in HCC, whereas cell surface expression of ULBP1 (but not MICA) was associated with improved recurrence-free survival (Kamimura et al. 2012). However there was a strong correlation between soluble MICA in the periphery and vascular invasion of the tumour (Kumar et al. 2012). MICA in this setting may not have a causative effect on survival, and may instead be confounded by some other consequence of vascularity. I set out to survey soluble NKG2D ligands in a cohort of hepatitis B-associated HCC and CHB patients in Africa, to investigate what process is likely to be driving their release, whether soluble NKG2D ligands influence survival in this setting and whether alterations to NK cells are likely to contribute to this effect.

3.2 Hypothesis and research questions

Hypothesis: HCC secretes NKG2D ligands into the circulation and these ligands affect survival via an effect on NK cell anti-tumour function mediated by NKG2D.

1. Which soluble NKG2D ligands are released by hepatitis B-associated HCC?

2. Does soluble ligand release affect NKG2D expression by circulating NK cells and if so, does this impact effector function?

3. Does NKG2D ligand expression have prognostic association and are these independent of disease staging and other known prognostic markers?

4. Is NKG2D ligand expression related to the aetiology of the HCC?

5. Is soluble NKG2D ligand expression seen in secondary liver tumours?

6. What is the mechanism by which NKG2D ligands are induced?

3.3 Patient cohort

Demographic and clinical data for this cohort is shown in Table 3.1. Patients were grouped according the criteria in Table 3.2. Cut-off values for viral load and ALT were the same as those used for clinical management. Note that some of my groups, particularly the cirrhosis and active CHB groups, patients have been included from both WATCH and HC4. The active CHB will contain both HBeAg positive chronic hepatitis and HBeAg negative chronic hepatitis patients. Management is similar in both disease stages. Similarly the cirrhosis group contains a range of disease activities, but is an important comparator for the HCC patients.

Table 3.1 Demographic and clinical characteristics of the CHB, HCC and control cohorts

Parameter	CHB	HCC	Healthy control
Male:Female ratio	92:30 (75.4%)	56:14 (80%) p=0.59	9:23 (28.1%) p<0.0001
Age (years)	35 (16-84)	43 (23-85) p=0.0042	53 (31-99) p<0.0001
HBV DNA (IU/ml)	255 (0-4.7x10^8)	1018 (0-3.2x10^{10}) p=0.29	-
ALT (IU/ml)	31 (9-1311)	60 (15-889) p=0.12	23 (13-54) p=0.0027
Platelet count (x10^9/l)	161 (16-1147)	270 (80-644) p<0.0001	208 (97-432) p=0.051
Albumin (g/dL)	40 (15-49)	32 (16-51) p<0.0001	43 (36-50) p<0.0001

Median (range) for age, HBV DNA viral load, alanine transaminase (ALT), platelet count and albumin of the chronic hepatitis B (CHB), hepatocellular carcinoma (HCC) and healthy control groups. Student's t-test with Welch's correction was used to calculate p values using the CHB cohort as a reference.

3.4 Results

3.4.1 Soluble ULBP1 is found in the serum of patients with HCC and active HBV

Serum from a cohort of patients with HBV-associated HCC, CHB in various clinical stages and controls was screened for the NKG2D ligands MICA, MICB, ULBP1, ULBP2 and ULBP3 (Fig 3.1 A-E). Sporadic individuals had detectable amounts of MICA and MICB, but there were no consistent differences between clinical groups. Very few were positive for ULBP2 or ULBP3 (Fig 3.1 A, B, D, E). However serum ULBP1 concentrations showed a clear, stepwise increase from inactive to active and cirrhotic CHB to HCC (Fig 3.1 C). Controls and patients with inactive CHB had low and similar levels of ULBP1, whereas these were significantly raised in patients with active CHB (defined as ALT>80 IU/ml or HBV DNA >2000 IU/ml) and in those with cirrhosis (median liver stiffness >10kPa or histologically proven cirrhosis). HCC patients had significantly higher levels of ULBP1 than all the CHB groups, with only 2 out of 34 individuals in this group below the limit of detection.

NKG2D ligands in serum may be present as soluble protein, either as a result of proteolytic cleavage or alternative splicing, or on circulating exosomes. This may affect their activity as multi-valent exosomes can more readily cross-link NKG2D than individual soluble proteins, leading to NKG2D downregulation on NK cells and impaired cytotoxicity (Fernández-Messina et al. 2010; Ashiru et al. 2010; Labani-Motlagh et al. 2016). In a subset of patients with high ULBP1 levels, serum was separated by size exclusion chromatography into exosome and protein fractions. ULBP1 concentrations were similar in the whole serum and protein fractions and significantly lower in the exosome fraction (Fig 3.2 A). The presence of exosomes in the exosome fraction was confirmed by staining of membranous bodies with BODIPY-FL and visualisation by imaging cytometry (Fig 3.2 B).

Table 3.2 Clinical criteria for patient groups.

Clinical Group	Criteria
HCC	HBsAg positive, HCC on histology or liver mass on USS and raised AFP
CHB Cirrhosis	HBsAg positive, MLS >10kPa or cirrhosis on histology
Active CHB	HBsAg positive, VL >2000 IU/ml or ALT >80 IU/ml
Inactive CHB	HBsAg positive, VL<2000 IU/ml, ALT<80 IU/ml
Negative control	HBsAg negative

Apart from the negative control group, all patients included in the NKG2D ligand serum survey were HBsAg positive. USS, ultrasound scan; MLS, median liver stiffness calculated by fibroscan; VL, hepatitis B virus DNA viral load in plasma.

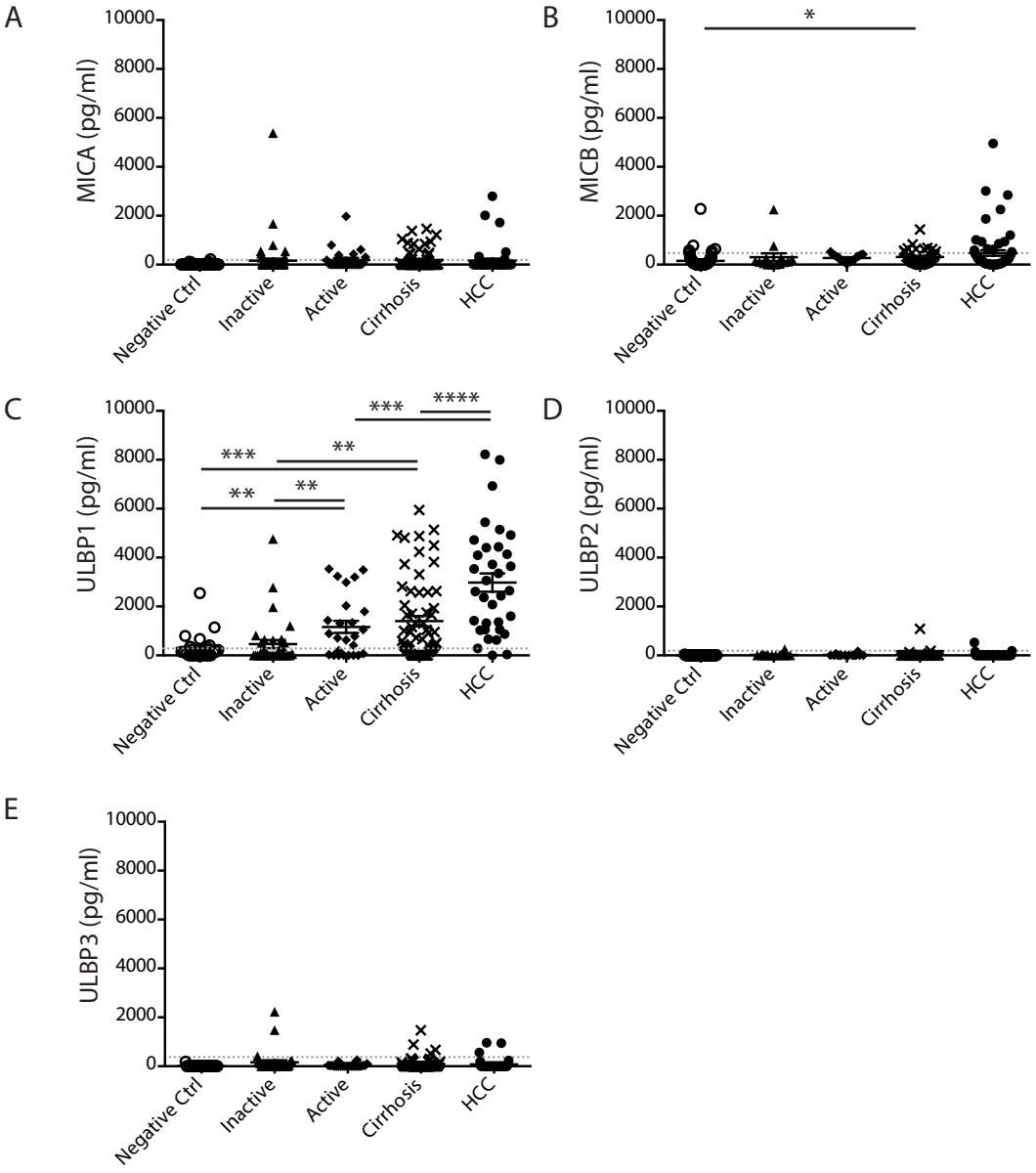

Figure 3.1 ULBP1 is elevated in the serum of individuals with HCC or advanced CHB disease

To form a holistic assessment of soluble NKG2D ligand expression in HCC and CHB, serum concentrations of soluble NKG2D ligands were measured by sandwich ELISA in five participant groups – HCC (HBV associated HCC), Cirrhosis (HBV associated cirrhosis defined as median liver stiffness >10kPa or biopsy-proven liver cirrhosis), Active (HBV without HCC or cirrhosis but with viral load >2000 IU/ml or ALT >80 U/l), Inactive (HBV without HCC, Cirrhosis, raised HBV viral load or raised ALT) and Negative control (HBsAg negative). Data shown for A. MICA (n=178), B. MICB (n=132), C. ULBP1 (n=177), D. ULBP2 (n=133), E. ULBP3 (n=181). Grey dotted lines indicate the limit of quantitation. Values below limit of quantitation were set to zero before statistical analysis by Mann-Whitney U test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

ULBP1 was demonstrated on a small proportion of exosomes, which were CD45 negative (Fig 3.2 C). This suggests that the majority of soluble ULBP1 in serum is present as soluble protein, rather than being expressed on a membranous body, although it is possible that some ULBP1 is shed by HCC on tumour exosomes.

3.4.2 Soluble ULBP1 does not affect NK cells

Other soluble NKG2D ligands, notably MICA, have been shown to impair NK cell function. To investigate whether serum ULBP1 had an affect on NK cell function, we first looked at NKG2D expression *ex vivo* by flow cytometry. NKG2D expression on both NK cells and CD8 T cells was not affected by either disease stage or by the presence of ULBP1 (Fig 3.3 A, B). In line with this *ex vivo* finding, recombinant human ULBP1 also did not affect NKG2D expression on NK cells when added *in vitro*, even at high concentrations (Fig 3.3 C).

3.4.3 In CHB, soluble ULBP1 is associated with viraemia

We looked at a number of clinical parameters that might drive ULBP1 production in the CHB cohort. There was no association between ULBP1 and sex or age amongst CHB patients (Fig 3.4 A, B). In keeping with the observation that ULBP1 is associated with active CHB, there was a correlation between HBV DNA viral load and ULBP1 levels (Fig 3.4 C). Taking the clinically-relevant cut-off of 2000 IU/ml, there was a highly significant difference between the ULBP1 levels of patients with high and low level viraemia (Fig 3.4 D). There was also a statistically significant association between serum ALT and ULBP1 concentrations in a univariate analysis. In keeping with the lack of significant difference between ULBP1 levels in cirrhotic CHB and active CHB without cirrhosis (Fig 3.1 C), there was no association between ULBP1 and three markers of cirrhosis: median liver stiffness, serum albumin or platelet count (Fig 3.5 A). In a multivariate

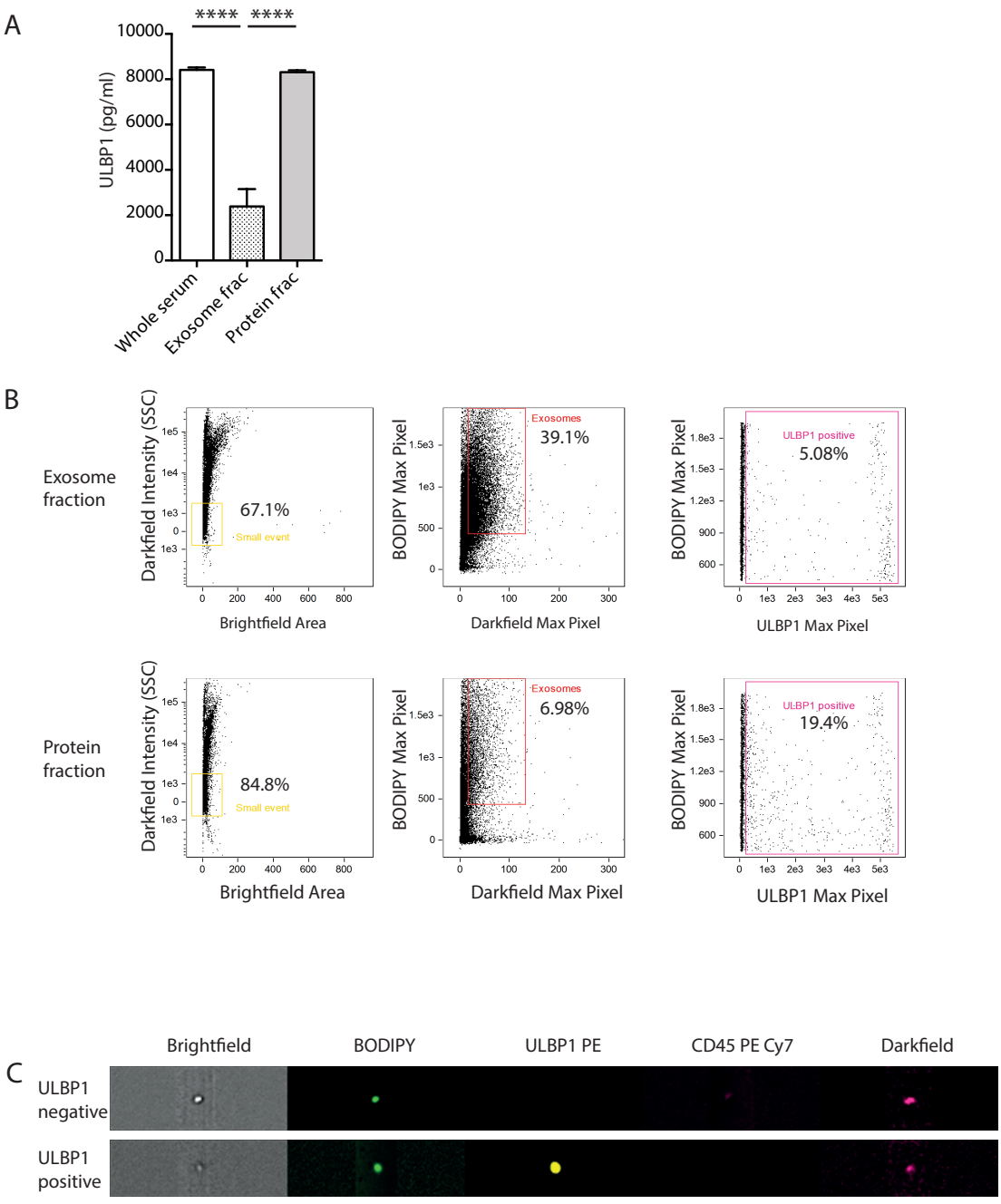

Figure 3.2 Serum ULBP1 is predominantly present as free protein rather than on exosomes

A. Serum was separated into exosome and protein fractions by size exclusion column filtration and ULBP1 concentration assessed by ELISA as in fig 1 (n=16). Groups were compared using Wilcoxon matched-pairs signed rank test.

B. Imaging cytometry of whole serum, protein and exosome fractions stained with BODIPY-FL and anti ULBP1-PE. Small events are then gated on BODIPY positive to find the exosomes, a proportion of which are ULBP1 positive.

C. Representative images of ULBP1 positive and negative exosomes from the serum of an HCC patient.

P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤ 0.005; ***, p ≤0.001; ****, p ≤0.0001.

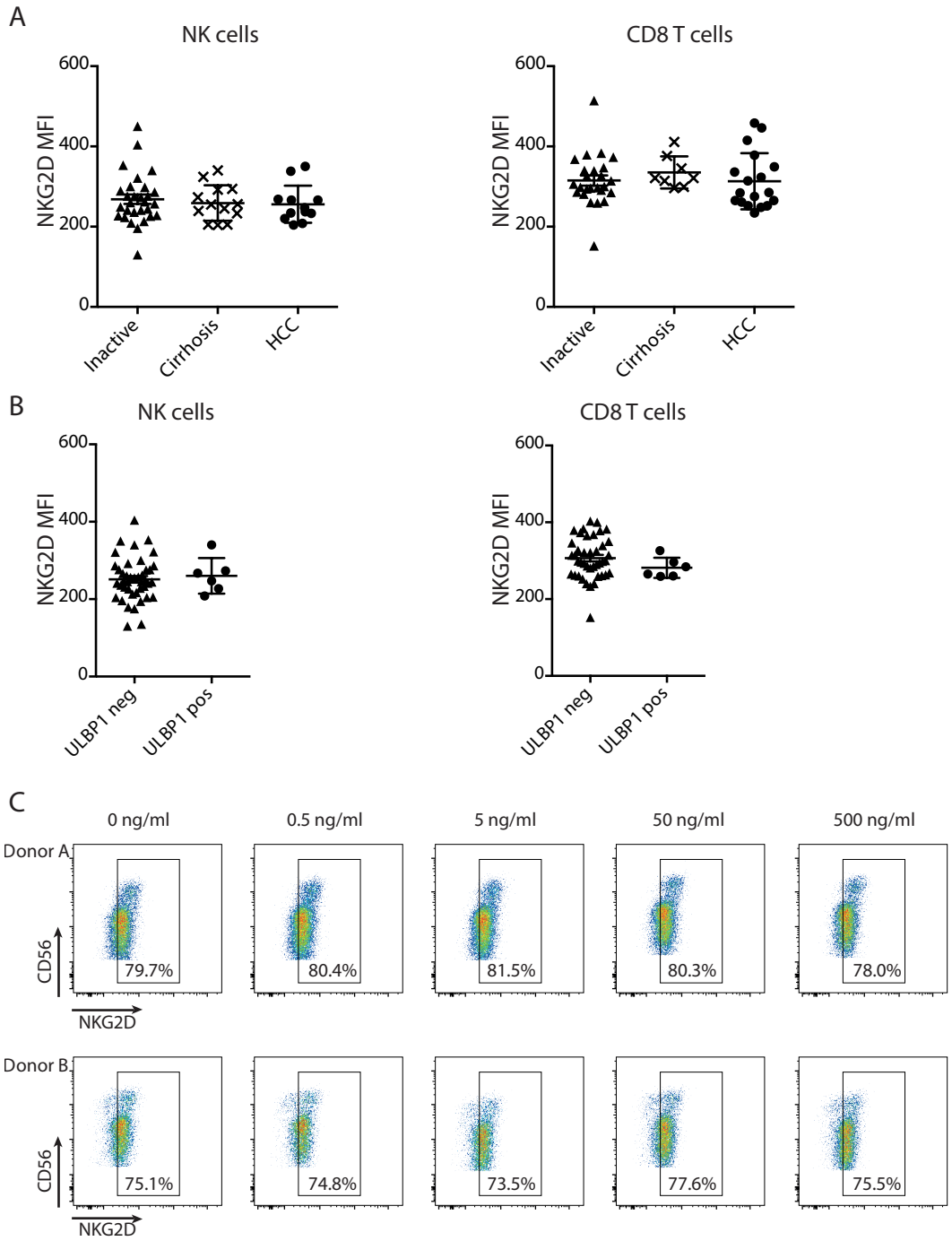

Figure 3.3 ULBP1 in serum does not affect the expression of NKG2D by NK cells or CD8 T cells.

A. NKG2D expression by flow cytometry and presented as MFI of Alexa 488 NKG2D on total NK cells (n=54) and CD8 T (n=51) cells in peripheral blood of individuals with HCC, HBV-associated cirrhosis and inactive CHB. Mean and SEM shown.

B. NKG2D expression by flow cytometry and presented as MFI of Alexa 488 NKG2D on total NK cells (n=48) and CD8 T (n=47) cells in peripheral blood of individuals with positive serum ULBP1 (above the limit of detection) and negative serum ULBP1. Mean and SEM shown.

C. NKG2D expression by flow cytometry on total NK cells from two healthy donors following 6 hour incubation with recombinant ULBP1 at increasing concentrations.

P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

analysis only viral load was found to be independently predictive of ULBP1 expression (Table 3.3). In a subset of patients, serum was available before and after 12 months of antiviral treatment with tenofovir. In this cohort the ULBP1 concentration fell significantly after treatment, which supports the association between viral replication and serum ULBP1 (Fig 3.4 F).

Using an *in vitro* model we investigated whether soluble ULBP1 release was due to a cell-intrinsic effect of HBV replication within hepatocytes. HepG2 AD38 and HepG2.117 are cell lines transfected with full-length HBV genomes under Tet-off promoters, so culture with doxycycline suppresses HBV replication. They secrete HBV virions at high and low titre respectively. ULBP1 was not seen in the culture supernatant of either cell line. HepG2 NTCP cells are HepG2 cells transfected with sodium taurocholate cotransporting peptide (NTCP), a bile transporter that is also the entry receptor for the HBV virion. This cell line is permissive for HBV infection but produces only very low numbers of viral particles. ULBP1 was detected at low level in 3 of 6 HBV infected HepG2 NTCP supernatants (Fig 3.5 B). Infection of primary human hepatocytes with HBV isolates from patients did not affect ULBP1 production, which was seen at high levels in both infected and uninfected cultures (Fig 3.5 C).

3.4.4 In HCC, soluble ULBP1 is independent of viraemia and has prognostic significance

In the HCC cohort, serum ULBP1 concentration was not correlated with HBV DNA viral load or serum ALT (Fig 3.6 A), or with markers of cirrhosis (serum albumin or platelet count, Fig 3.6 B) Median liver stiffness is not a measure of fibrosis/cirrhosis in this setting as the stiffness of the tumour exceeds that of the liver. Tumour volume can be estimated from the two linear dimensions reported on ultrasound scan of the liver(Sapi et al. 2015). Using this approach, ULBP1 was not associated with tumour volume (Fig 3.6 C).

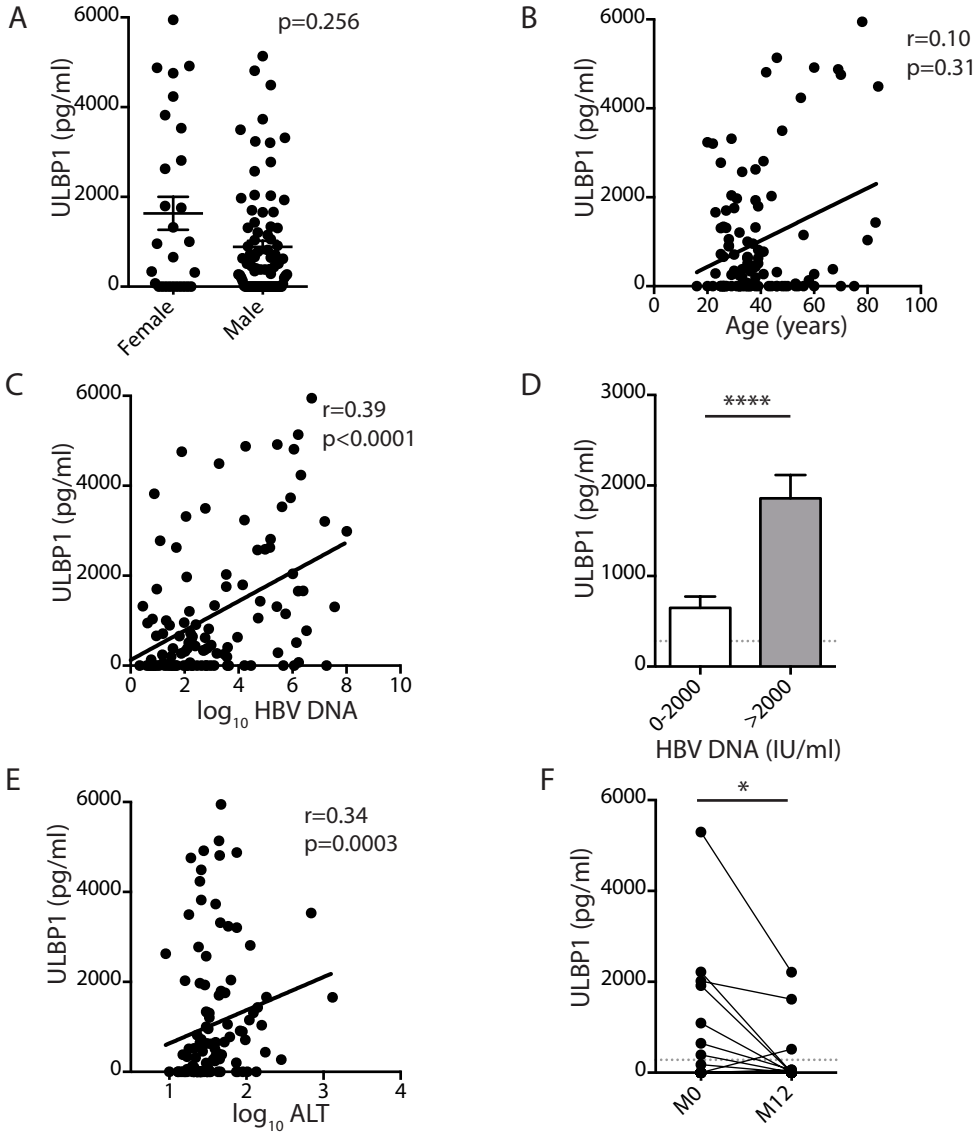

Figure 3.4 In CHB, ULBP1 is associated with HBV replication and falls following treatment

A. Serum ULBP1 concentrations measured by ELISA in male and female individuals with CHB (n=111).

B. Serum ULBP1 concentrations measured by ELISA in the CHB individuals against age (n=111).

C. Serum ULBP1 concentrations measured by ELISA in the CHB individuals against log10 HBV DNA concentration (n=111).

D. Serum ULBP1 concentration measured by ELISA for the CHB individuals (Cirrhosis, Active and Inactive) grouped by viral load above and below 2000 IU/ml (n=111).

E. Serum ULBP1 concentrations measured by ELISA in the CHB individuals against log10 serum ALT concentration (n=113).

F. Serum ULBP1 concentrations measured by ELISA at baseline (M0) and after 12 months of treatment with tenofovir (M12) (n=50).

Groups compared using Wilcoxon matched-pairs signed rank test (paired) and Mann-Whitney U test (unpaired). P-values for correlations calculated by Spearman nonparametric correlation. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤ 0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

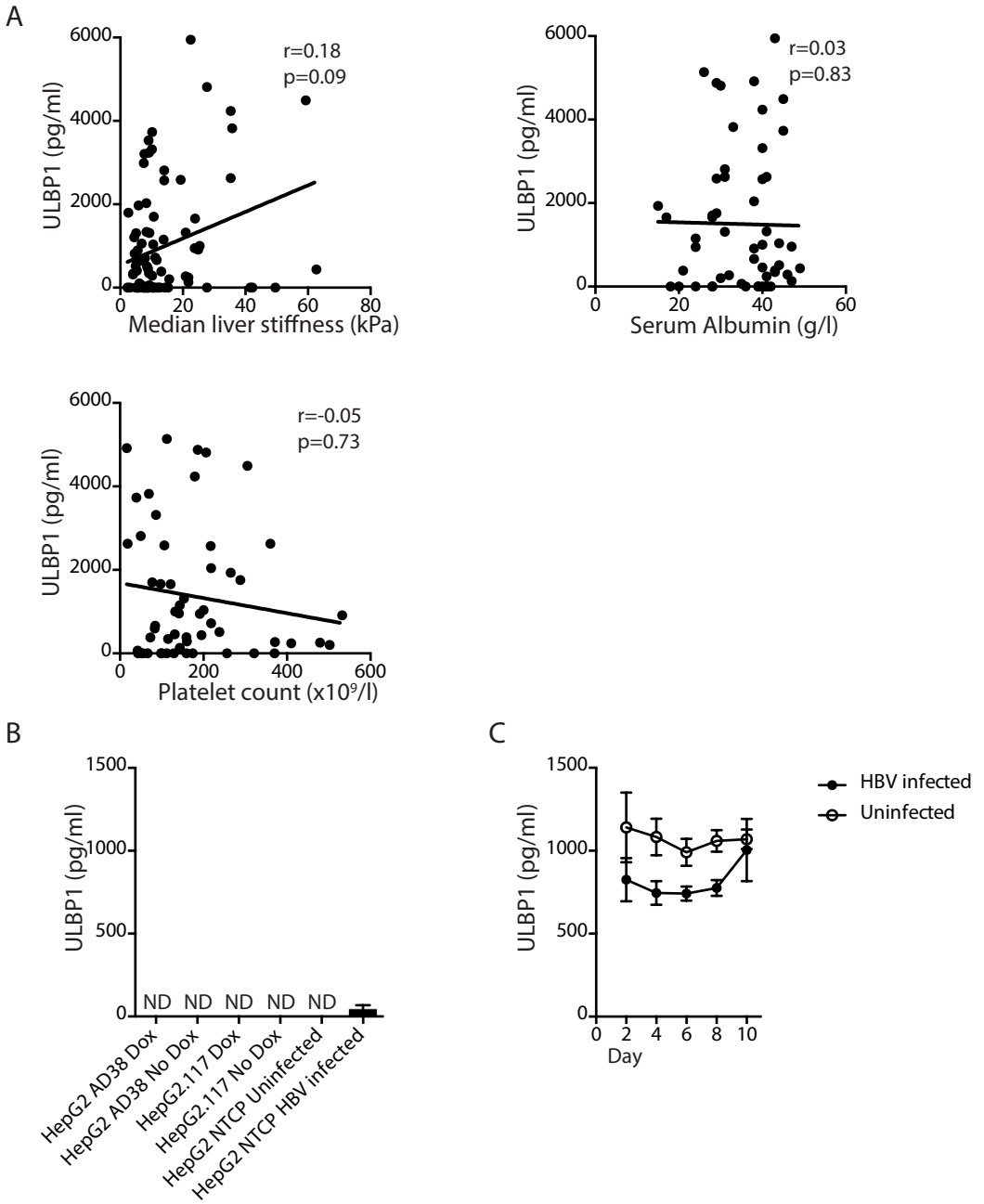

Figure 3.5 ULBP1 is not associated with markers of fibrosis, and is not produced by *in vitro* HBV replication

A. Serum ULBP1 concentrations measured by ELISA in individuals with HBV-associated liver cirrhosis against median liver stiffness (n=87), serum albumin concentration (n=55) and platelet count (n=56). P-value calculated by Spearman nonparametric correlation.

B. ULBP1 concentration measured by ELISA in 3-day culture supernatant from HepG2 AD38 (n=3 for each group) and HepG2.117 (n=3 for each group) cells with and without doxycycline (inhibitor of HBV gene expression), and from HBV infected and control HepG2 NTCP cells (n=6 for each group). Mean and SEM shown. ND, not detected.

C. ULBP1 concentration measured by ELISA in supernatant from HBV infected and paired control primary human hepatocyte cultures (n=4 for each group). Mean and SEM shown.

Table 3.3 Multivariate analysis of predictors of serum ULBP1 concentration in CHB patients

Parameter	Coefficient	Standard Error	p value	95% confidence interval
ALT	-1.7	3.02	0.593	-7.62 - 4.38
Log HBV DNA	302.0	65.47	>0.0001	172.0 - 431.9
Age 0-40 years	0	-	-	-
Age 41-60 years	477.8	296.7	0.111	-111 - 1067
Age 61+ years	1553	452.5	0.001	655.0 - 2451
Female	0	-	-	-
Male	-394.8	300.5	0.192	-991.4 - 201.7

Coefficient represents the predicted change in serum ULBP1 concentration (pg/ml) of an increase in one unit of the named parameter (1IU ALT or 1 log HBV DNA) or with reference to the base group (age, male vs female). Coefficient, standard error, p value and confidence intervals calculated by multiple logistic regression.

We sought to understand whether soluble ULBP1 production was a property of all intrahepatic tumours or just of hepatitis B-associated HCC. ULBP1 concentrations were much higher in the serum of patients with HBV-associated HCC compared with patients with secondary liver tumours of various causes (cholangiocarcinoma, lymphoma, metastatic pancreatic and colorectal cancer) (Fig 3.7 A). To confirm ULBP1 release direct from HCC, small pieces of tumour were weighed and incubated for 18 hours in 10x their mass of cell culture medium. Conditioned media was removed and centrifuged to remove all cells and debris before ELISA. ULBP1 was detectable in conditioned media and ULBP1 concentrations were higher in HCC conditioned media than in conditioned media from metastases of colorectal cancer (CRC) (Fig 3.7 B). In a UK-based cohort of HCC of various causes, there was no difference in soluble ULBP1 concentrations between HCC caused by HBV, HCV or other causes (Fig 3.7 C). This suggests that HCC, irrespective of aetiology, drives ULBP1 expression. ULBP1 levels were lower in HCC patients from the UK than from the Gambia (Fig 3.7 D), which may be indicative of a clinical difference between these groups.

There was a strong association between serum ULBP1 concentration and survival. When ULBP1 concentration at first clinic visit was plotted against time from first clinic visit to death, a negative association was seen, indicating high ULBP1 concentration is associated with short survival (Fig 3.8 A). This may account for the difference between the ULBP1 concentrations in Gambian and UK patients, as patients in The Gambia tend to present late and have very poor prognosis, median survival in this cohort was 34 days. Comparing ULBP1 concentrations above and below 2000pg/ml, the Kaplan-Meier curves showed a highly significant difference in survival (Fig 3.8 B), with a median survival of 76 days in the 0-2000pg/ml group and 22 days in the >2000pg/ml group. Other parameters known to have prognostic significance in HCC include age, serum albumin, bilirubin, alkaline phosphatase, tumour volume, the presence of ascites, and WHO

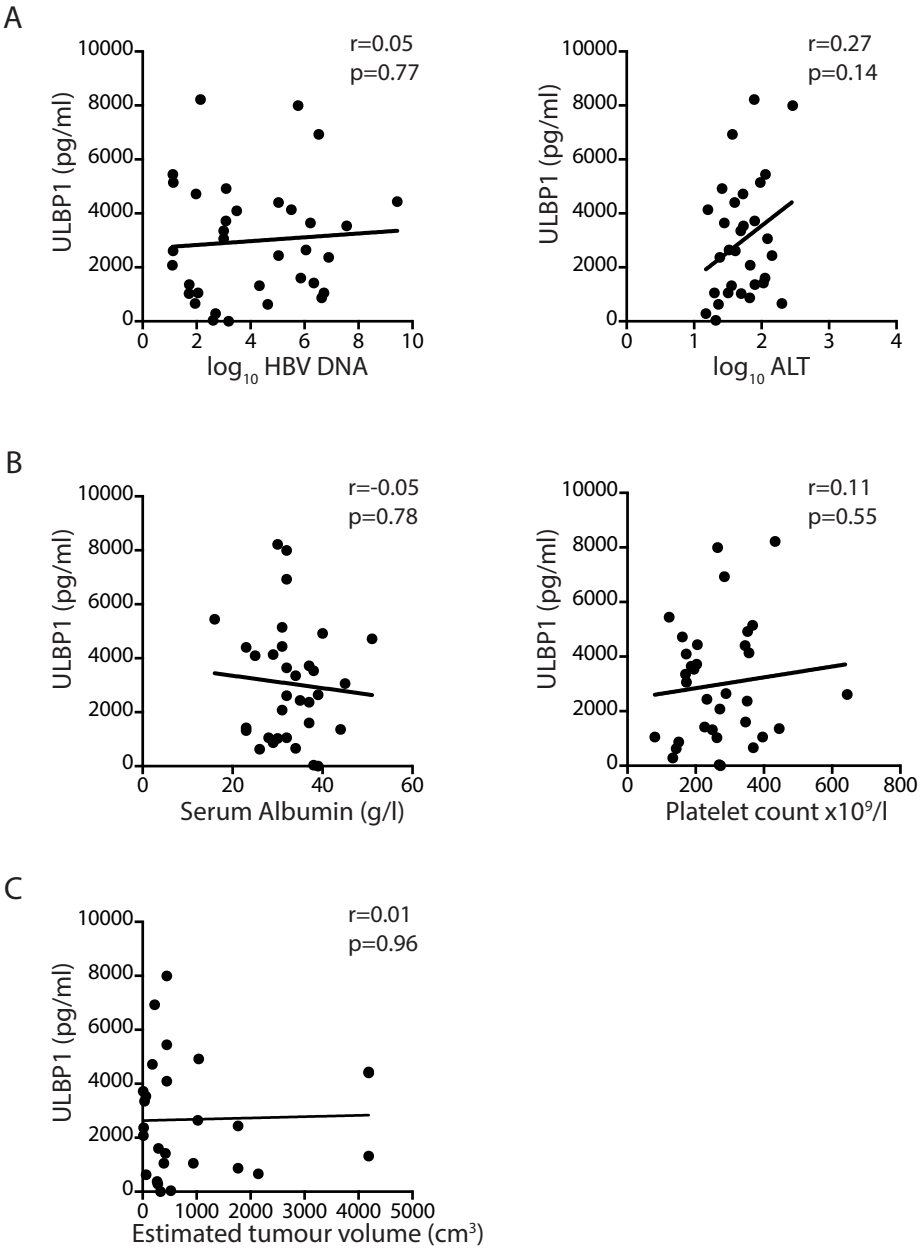

Figure 3.6 In HCC, ULBP1 is not associated with viral replication or tumour burden
A. Serum ULBP1 concentrations measured by ELISA in individuals with HCC against log10 HBV DNA concentration (n=34) and log10 serum ALT concentration (n=31).
B. Serum ULBP1 concentrations measured by ELISA in individuals with HCC against serum albumin (n=33) and platelet count (n=34).
C. Serum ULBP1 concentrations measured by ELISA in individuals with HCC against tumour volume, estimated by calculating the volumes of spheroids with dimensions measured by liver ultrasound scan (n=27).
P-values for correlations calculated by Spearman nonparametric correlation.

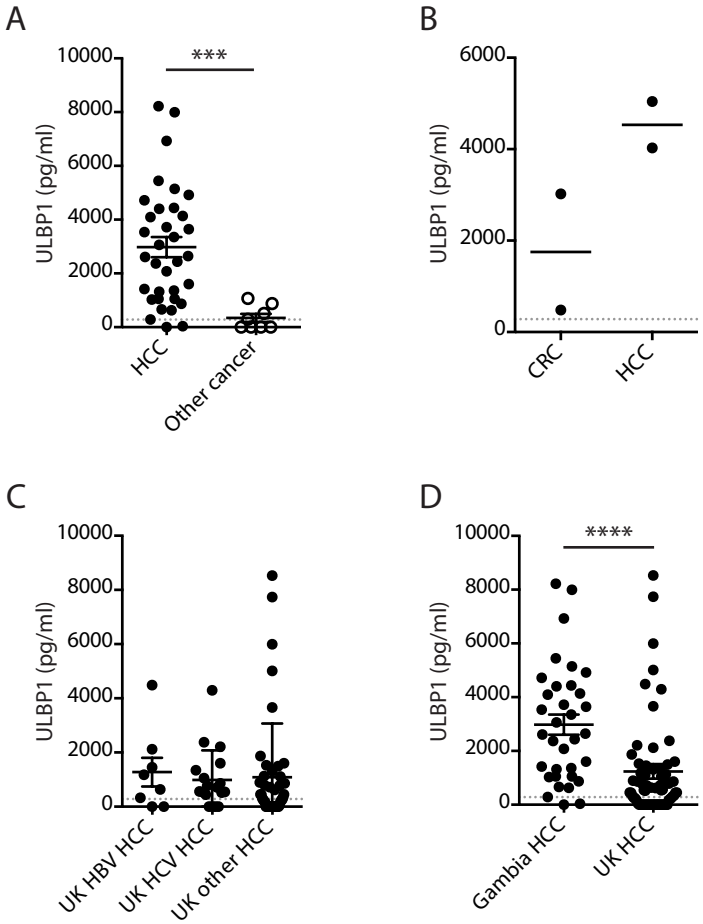

Figure 3.7 ULBP1 is present at high levels in serum from HCC patients but not in serum from secondary liver cancer patients

A. Serum ULBP1 concentration measured by ELISA in HBV-associated HCC (n=34), and non-HCC secondary tumours within the liver (n=8).

B. ULBP1 concentration measured by ELISA in overnight conditioned media from CRC and HCC tissue (n=2 in each group).

C. Serum ULBP1 concentration measured by ELISA in HBV-associated HCC (n=8), HCV-associated HCC (n=18) and HCC of other causes (n=45), all from a UK cohort.

D. Serum ULBP1 concentration measured by ELISA in HCC patients from The Gambia (n=34), and from the UK (n=71).

Groups compared by Mann-Whitney U test. P-values for correlations calculated by Spearman nonparametric correlation. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

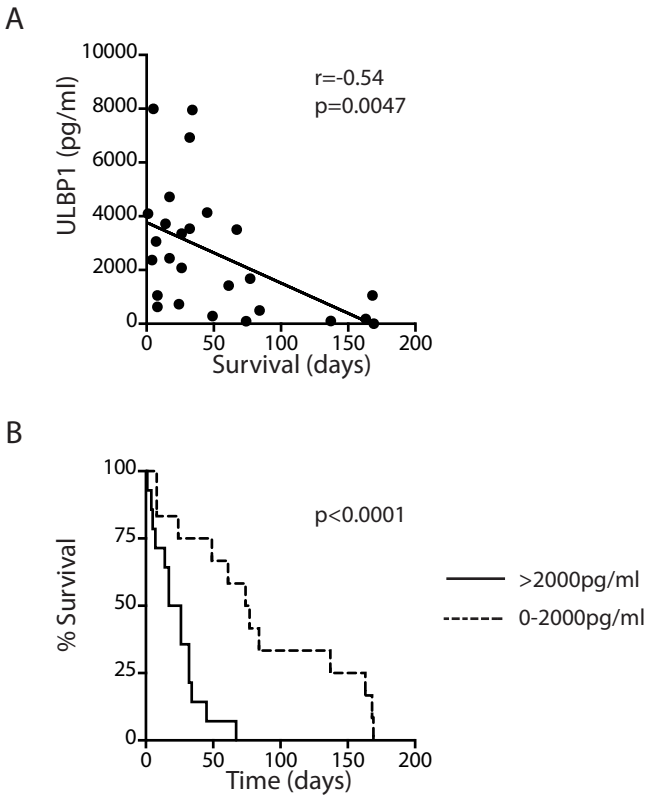

Figure 3.8 Serum ULBP1 predicts survival with HCC in The Gambia
A. Soluble ULBP1 measured by ELISA at baseline clinical assessment in individuals with HCC plotted against survival (n=29). P-value for correlation calculated by Spearman nonparametric correlation.
B. Kaplan-Meier curves comparing survival from the day of baseline clinical assessment between groups with serum ULBP1 concentration measured by ELISA above and below 2000pg/ml. P-value for difference calculated by log-rank test.

performance status (op den Winkel et al. 2012; Hsu et al. 2013). In univariate analysis, ULBP1>2000pg/ml had a hazard ratio for death of 3.65, comparable to that of bilirubin, albumin and ascites (Table 4). WHO performance status of 2 or greater and Okuda stage 3 (Okuda et al. 1985) were also positively associated with mortality, consistent with other cohorts. Age and sex did not have a significant effect on prognosis, which was poor among all demographic groups.

We used Poisson regression to compare prediction models with and without ULBP1 concentration. ULBP1 was an independent predictor of survival in this cohort when age, sex, WHO performance status and Okuda stage (a clinical scoring system incorporating ascites, albumin, bilirubin and disease burden) were accounted for (Table 5), suggesting that serum ULBP1 measurement gives additional prognostic information not captured by these other measures. In an alternative model separately incorporating ULBP1, age, sex, simplified WHO performance status, bilirubin, albumin and ascites showed no significant associations with survival (the large number of covariates decreasing statistical power), but ULBP1 had a hazard ratio less than that of bilirubin but comparable to ascites, albumin and performance status (Table 6).

Table 3.4 Univariate associations with mortality in HBV-associated HCC patients

Parameter and cutoff	Hazard ratio	p value
Bilirubin >50µmol/l	5.18 (1.934 - 13.873)	0.0003
ULBP1 >2000pg/ml	3.65 (1.687 - 7.883)	0.0004
Albumin ≤30g/dL	3.46 (10479 - 8.076)	0.0023
Ascites	2.66 (1.229 - 5.745)	0.0098
Alkaline phosphatase >150IU/L	2.38 (0.889 - 6.373)	0.075
WHO performance status 2+	2.73 (1.193 - 6.227)	0.017
Okuda stage 1	1	-
Okuda stage 2	1.43 (0.583 - 3.528)	0.433
Okuda stage 3	4.19 (1.403 - 12.493)	0.010
Age 0-40	1	-
Age 41-60	0.59 (0.263 - 1.304)	0.190
Age 61+	2.44 (0.323 - 18.495)	0.387
Female sex	1.804 (0.677 - 4.805)	0.231

Hazard rate ratio for death and p value calculated by linear regression for ULBP1 and a range of clinical parameters. WHO performance status was grouped into 0-1 and 2-4. Ascites was defined by ultrasound.

Table 3.5 Multivariate analysis of mortality in HBV-associated HCC, incorporating Okuda stage

Parameter	Hazard ratio (95% confidence interval)	p value	Hazard ratio in multivariate	p value in multivariate
ULBP1 >2000	3.65 (1.69-7.88)	0.0012	5.23 (1.49 - 18.37)	0.01
Okuda stage 1	1	0.066		
Okuda stage 2	1.43 (0.58-3.53)			
Okuda stage 3	4.19 (1.40-12.49)			
WHO performance status 2-4	2.73 (1.19-6.23)	0.015	1.66 (0.63 - 4.38)	0.309
Age 0-40 years	1	0.26	1	0.85
Age 41-60 years	0.58 (0.26-1.3)		1.36 (0.46 - 4.07)	
Age 61+ years	2.44 (0.32-18.50)		0.93 (0.12 - 7.51)	
Female	1.8 (0.68-4.81)	0.27	0.48 (0.14 - 1.63)	0.24

Hazard rate ratio for death and p value calculated by linear regression for ULBP1 and a range of clinical parameters. Parameters with an overall p value <0.05 as well as age and sex were taken forward into a Poisson regression model to calculate multivariate hazard rate ratios for death and associated p values.

Table 3.6 Multivariate analysis of mortality in HBV-associated HCC, incorporating ascites, bilirubin and albumin

Parameter	Hazard ratio (95% confidence interval)	p value	Hazard ratio in multivariate	p value in multivariate
ULBP1 >2000	3.65 (1.69-7.88)	0.0012	1.78 (0.4 - 8.00)	0.45
Bilirubin >50µmol/l	5.18 (1.934 - 13.873)	0.0003	3.02 (0.76 - 11.95)	0.12
Ascites	3.46 (10479 - 8.076)	0.0023	1.68 (0.44 - 6.08)	0.94
Albumin ≤30g/dL	2.66 (1.229 - 5.745)	0.0098	1.68 (0.44 - 6.51)	0.45
WHO performance status 2-4	2.73 (1.19-6.23)	0.015	1.6 (0.42 - 6.08)	0.49
Age 0-40 years	1	0.26	1	0.73
Age 41-60 years	0.58 (0.26-1.3)		0.74 (0.19 - 2.82)	
Age 61+ years	2.44 (0.32-18.50)		2.02 (0.21 - 19.55)	
Female	1.8 (0.68-4.81)	0.27	0.48 (0.14-1.63)	0.24

Hazard rate ratio for death and p value calculated by linear regression for ULBP1 and a range of clinical parameters. Parameters with an overall p value <0.05 as well as age and sex were taken forward into a Poisson regression model to calculate multivariate hazard rate ratios for death and associated p values.

3.5 Discussion

Previous work on soluble NKG2D ligands in HCC has focused on MICA. We present the first survey of MICA, MICB, and ULBP 1, 2, 3. Due to the sequence homology between ULBPs 2, 5 and 6, the ULBP2 ELISA also detects ULBP5 at 0.5% cross-reactivity and ULBP6 at 27.9% cross-reactivity. As ULBP2 was undetectable in 129 of 133 individuals it is unlikely that ULBP5 or 6 are present in significant amounts in the serum of CHB or HCC patients. This is also the first study of NKG2D ligands outside of Asia. Previous series from Japan (Kumar et al. 2012) and China (J.-J. Li et al. 2013) demonstrated the presence of soluble MICA in a proportion of individuals with HCC, and that this ligand was associated with a poor prognosis. Similar mechanisms have been shown for MICA in prostate (Wu et al. 2004) and colorectal cancer (Doubrovina et al. 2003) and for ULBP2 in ovarian cancer (K. Li et al. 2008) and malignant melanoma (Paschen et al. 2009).

Our patient cohort was nested within the Prolifica study, which incorporates a community screened cohort of chronic hepatitis B (CHB) patients over 30 years of age (Lemoine et al. 2016) and a cohort of individuals referred with signs and symptoms of liver disease, who were assessed for HCC. Hepatitis B negative controls were derived exclusively from the community screening, and were older than either of the disease groups, possibly because older individuals were more willing to attend the MRC Unit. Compared to the CHB cohort, the HCC group was older, with similar HBV DNA viral load and ALT but with a higher platelet count and lower albumin, likely indicative of a catabolic and inflammatory response to the tumour (Porporato 2016; Carr & V. Guerra 2013).

In this cohort of patients with hepatitis B from The Gambia, elevated MICA was observed in only a small number of individuals with HCC. By contrast ULBP1 was elevated in almost all of our cohort with HBV-related HCC.

ULBP1 was also raised, to a lesser extent, in the serum of CHB patients with cirrhosis or active hepatitis B disease without HCC. Overall ULBP1 levels were similar between hepatitis B-negative controls and those with inactive hepatitis B, effectively dividing the five clinical categories into three groups by ULBP1: HCC; cirrhotic and active CHB; inactive CHB and HBV negative individuals.

NKG2D ligands can be expressed and released from cells in a variety of ways, which may have consequences for NKG2D signalling and downstream functional responses (Raulet et al. 2013). In particular NKG2D ligands shed on exosomes have the potential to modulate NK cell lytic function. In this cohort, ULBP1 was not shed primarily as exosomes but as soluble protein. This may explain the lack of effect on NKG2D expression on *ex vivo* NK cells and CD8 T cells, supported by the preservation of NKG2D on the surface of NK cells in the face of increasing concentrations of recombinant human ULBP1. This is in contrast to the impairment of NKG2D expression and function seen in settings of soluble MICA (Groh et al. 2002; Jinushi et al. 2005). It is known that ULBP2 is a weaker downregulator of NKG2D on NK cells than MICA despite similar binding affinities, the explanation for this is unclear, and may be related to the spatial organisation of receptor microclusters (Molfetta et al. 2014).

In chronic hepatitis B disease, NK cells may play multiple roles - reviewed in (Maini & Peppa 2013). NK cells may contribute to viral control by killing infected hepatocytes (Dunn et al. 2007), may inhibit fibrosis by killing intrahepatic stellate cells (Glassner et al. 2012), and can modulate T cell responses via TRAIL (Peppa et al. 2013) and NKG2D (W.-C. Huang et al. 2017). In most cases these effects are most pronounced in patients with active inflammation. Although ALT is positively correlated with ULBP1 concentration, in multivariate analysis only HBV DNA viral load and age over 61 years were independently associated with ULBP1, and specific antiviral

treatment that inhibits viral replication within 12 months reduces serum ULBP1 (Gordon et al. 2013). It is unclear why advanced age is associated with raised ULBP1, especially as when considered as a continuous variable there is no significant correlation. It may be that older individuals are more likely to have microscopic or preclinical hepatomas and are therefore behaving like individuals in the HCC group. Alternatively it may be an effect of the unusual clinical course of CHB, where a high viral load in young and old individuals may represent different disease stages (Rehermann & Nascimbeni 2005).

HBV replicating hepatoma cell lines do not release significant amounts of ULBP1, suggesting that ULBP1 may not be produced as a direct consequence of HBV infection of hepatocytes. It may be that interaction with another cell type is required to induce ULBP1 production in response to HBV replication, and this may also explain the wide variation in serum ULBP1 even in those patients with elevated HBV DNA viral load. Alternatively this may be a consequence of the relatively low level of viral replication seen in even the best *in-vitro* culture systems compared to the *in vivo* state. Interestingly, primary human hepatocytes secreted soluble ULBP1 irrespective of HBV infection, which may be indicative of some cellular stress related to the specialised culture system required to maintain these cells (March et al. 2015).

By contrast to CHB, in HCC HBV DNA viral load is not a predictor of ULBP1 concentration, neither is ALT, tumour size or any of the measures of fibrosis. This is somewhat surprising – in Li et al there was an association between soluble MICA and tumour size (J.-J. Li et al. 2013) and in Kumar et al between MICA and tumour vascularity (Kumar et al. 2012). We were unable to consider tumour vascularity as we did not have access to Doppler ultrasound, but were able to estimate tumour volume as spheroids from two-dimensional ultrasound measurements, and over a wide range of volumes

there was no association with ULBP1 concentration. This may be because HCC presents late, with advanced disease in Africa (Gyedu et al. 2014). As a result patients with very large tumours often have large, single, slow growing lesions that may be less aggressive. It is unclear what the driver of this HBV-independent ULBP1 production is in HCC, it may be driven by integration of HBV genomes in HCC cells, leading to the dissociation of ULBP1 from HBV viral load, but we were not able to see evidence to support this in transfected HepG2 cells (Fig 3.5 B). Indeed, in a cohort of HCC patients with various aetiologies from the UK, raised ULBP1 was seen in HBV-associated, HCV-associated and non-viral HCC, suggesting HBV infection is not required to drive ULBP1 expression (Fig 3.7 C). ULBP1 was seen only at very low levels in non-HCC liver tumours in the Gambia, suggesting that the very high levels of ULBP1 seen in this cohort may not be due to an ethnic or local exposure effect or simply due to hepatocyte destruction and instead that soluble ULBP1 is associated with malignant hepatocytes. ULBP1 was detectable in conditioned media from HCC and CRC, but at a higher level in HCC, confirming this specificity.

It may be that in CHB, active or cirrhotic disease results in microhepatoma formation (Mason et al. 2016), which results in ULBP1 production, and that other active liver diseases may have similar effects.

ULBP1 was strongly associated with survival (Fig 3.8 A, B). Serum ULBP1 concentration >2000pg/ml was associated with a median survival of 22 days, compared with 76 days where ULBP <2000pg/ml. In univariate analysis the hazard rate ratio for death was 3.65 for HCC patients with ULBP1>2000pg/ml. This effect was independent of age, sex, Okuda stage or simplified WHO performance status (WHO performance status on a 0-5 scale was not significantly associated with survival in univariate analysis) in a Poisson regression model. In a large study of prognostic indicators in a cohort from Germany, Okuda staging was shown to be a good

prognostication method, and no single parameter had a hazard ratio for death above 1.7 (op den Winkel et al. 2012). Many of the German patients had some form of treatment, so there will be significant differences between the two cohorts. However, in univariate analysis the parameters associated with mortality were very similar (bilirubin, albumin, ascites), and the hazard ratio for death for ULBP1 was comparable to these well recognised prognostic markers, indicating that ULBP1 may be a clinically useful prognostic marker in advanced HCC. The highest concordance index for any method of HCC prognostication in this German cohort was 0.71 (values for concordance index range from 0.5-1), indicating some factors affecting prognosis are not captured in any of the available clinical scores. It was not possible to compare sULBP1 concentrations across histological grades as only a minority of patients had a tissue diagnosis – most HCC diagnoses both in the UK and in the Gambia were made on the basis of characteristic radiological features so histological grading is unavailable in most cases. To demonstrate the generalisibility of the association between serum ULBP1, work is underway to gather survival data from the UK cohort so that outcomes with high and low serum ULBP1 can be compared as for the Gambian cohort. A larger study of ULBP1 in HCC prognostication with a range of disease stages and grades will be required to assess whether this marker may be of clinical utility in this setting. It would be particularly important to ascertain whether elevated ULBP1 in CHB (or other liver diseases) is predictive of future HCC, or whether ULBP1 screening may improve on AFP as an early biomarker for HCC.

Chapter 4 NK cells infiltrating primary and secondary liver tumours

4.1 Introduction

NK cells are capable of providing both tumour surveillance and tumour immunity (Morvan & Lanier 2016). However, for a tumour to become clinically apparent, these protective mechanisms must have failed. If we could understand why NK cells fail to clear certain malignancies it might be possible to intervene, to re-activate NK cell-mediated tumour immunity as a form of cancer therapy. This might be of particular use in the liver, where NK cells are plentiful and both primary and secondary tumours are common.

The NKG2D receptor-ligand system is one of the best characterised of the activating NK cell receptors. NKG2D ligands are known to be expressed on HCC and colorectal tumours, where their loss is associated with a poor prognosis (Kamimura et al. 2012; McGilvray et al. 2009). Although NKG2D has been shown to be important in tumour surveillance in mice, it has also been associated with more rapid progression of HCC (N. Guerra et al. 2008; Sheppard et al. 2017). In humans it has been presumed that NKG2D ligand loss is a mechanism by which tumours can escape NKG2D-mediated surveillance by NK cells, although direct evidence for this is lacking (Mondelli 2012; Zhang et al. 2015). There are also conflicting data on the relative effects of membrane bound and soluble NKG2D ligands, with some models showing a protective effect of membrane bound ligands (G. Liu et al. 2013), whereas others show a protective effect for soluble NKG2D ligands (Deng et al. 2015). Similarly, some clinical settings show NKG2D ligand expression on the tumour surface can be associated with poor prognosis (McGilvray et al. 2010; Madjd et al. 2007).

We are grateful that an ongoing collaboration with the hepatobiliary surgical team at the Royal Free Hospital affords us access to resected liver and

tumour tissue from HCC and colorectal cancer patients. By examination of the NK cell populations at the site of malignancy and in the host organ distant to the tumour, we aimed to understand the nature of NK cell functional impairment in HCC and CRC liver metastases, and whether NKG2D function is altered in tumour-infiltrating NK cells in these malignancies. We also sought to examine whether NK cell function can be improved through engagement or blockade of the NKG2D receptor, or whether NKG2D-mediated NK cell function might be improved by other means.

In this setting it is important to examine the relative contributions of liver resident and non-resident, liver-infiltrating NK cells. There is a wide variation in their proportions between individuals, from 10-90% of the intrahepatic NK cell population (Stegmann et al. 2016). Previous work on liver NK cells in tumours have considered bulk NK cells compared to peripheral NK cells (Cai et al. 2008). If there are differences in the way these two populations respond to the tumour environment this may have important consequences for potential NK cell-based therapies.

4.2 Hypothesis and research questions

Hypothesis: NK cells infiltrating HCC have reduced anti-tumour function due to interactions in the tumour microenvironment mediated by NKG2D/NKG2D ligands and NKG2D function can be improved by manipulation of the NKG2D pathway.

1. Do NK cells infiltrating HCC resemble circulating or intrahepatic NK cells?

2. Do tumour-infiltrating NK cells have altered NKG2D expression?

3. Do tumour-infiltrating NK cells have altered effector function?

4. Does exposure to HCC in vitro alter NK cell NKG2D expression or effector function?

5. Can NK cell effector function be protected from the effect of HCC by targeting the NKG2D pathway?

6. Can other pathways be used to maintain NK cell effector function in the context of HCC?

4.3 Results

4.3.1 NK cells are prevalent but dysfunctional in human liver tumours *ex-vivo*

Studies of the immune infiltrate of HCC have typically focused on CD8 T cells rather than NK cells, with reviews making statements such as “TILs in HCC tissues are mainly T cells” (Shirabe et al. 2010) or “CD8+ T cells represent the predominant population within the tumour-infiltrating lymphocytes” (Schmidt & Thimme 2016). In order to determine whether NK cells make up a substantial proportion of the immune infiltrate of liver tumours, we compared their frequency with those of NKT cells (CD3+ CD56+), CD8 T cells (CD3+ CD56- CD8+) and CD8 negative conventional T cells (CD3+ CD56- CD8-) in blood, healthy liver margins and HCC or colorectal metastases. Cells were identified by multiparameter flow cytometry of single cell suspensions derived from liver and tumour tissue as shown in Fig 4.1 A. In both the primary liver tumour hepatocellular carcinoma (HCC) and secondary liver tumours arising from colorectal cancer (CRC) NK cells were prevalent, accounting for around 25% of the CD45+ lymphocytes, with a frequency intermediate between blood and liver. In both settings NK cell and CD8 T cell proportions were similar, with mean proportions of 21.5% and 19.1% for NK and CD8 T cells in HCC, and 27.0% and 19.6% for NK and CD8 T cells in CRC (Fig 4.1 B). In one example of blood, primary colonic adenocarcinoma, liver metastasis and liver tissue from a single patient, the composition of the CD45+ lymphocyte pool in the liver metastasis was more similar to that of the liver than to the peripheral blood or the primary tumour (Fig 4.1 C).

In a paired analysis, NK cells in tumours comprised a slightly lower proportion of the CD45+ lymphocyte pool than in unaffected liver tissue (Fig 4.2 A). However NK cells were detectable in all liver tumour infiltrates and constituted up to a maximum of 49% in HCC and 55% in CRC metastases. Recent work from our laboratory and others has demonstrated a liver-

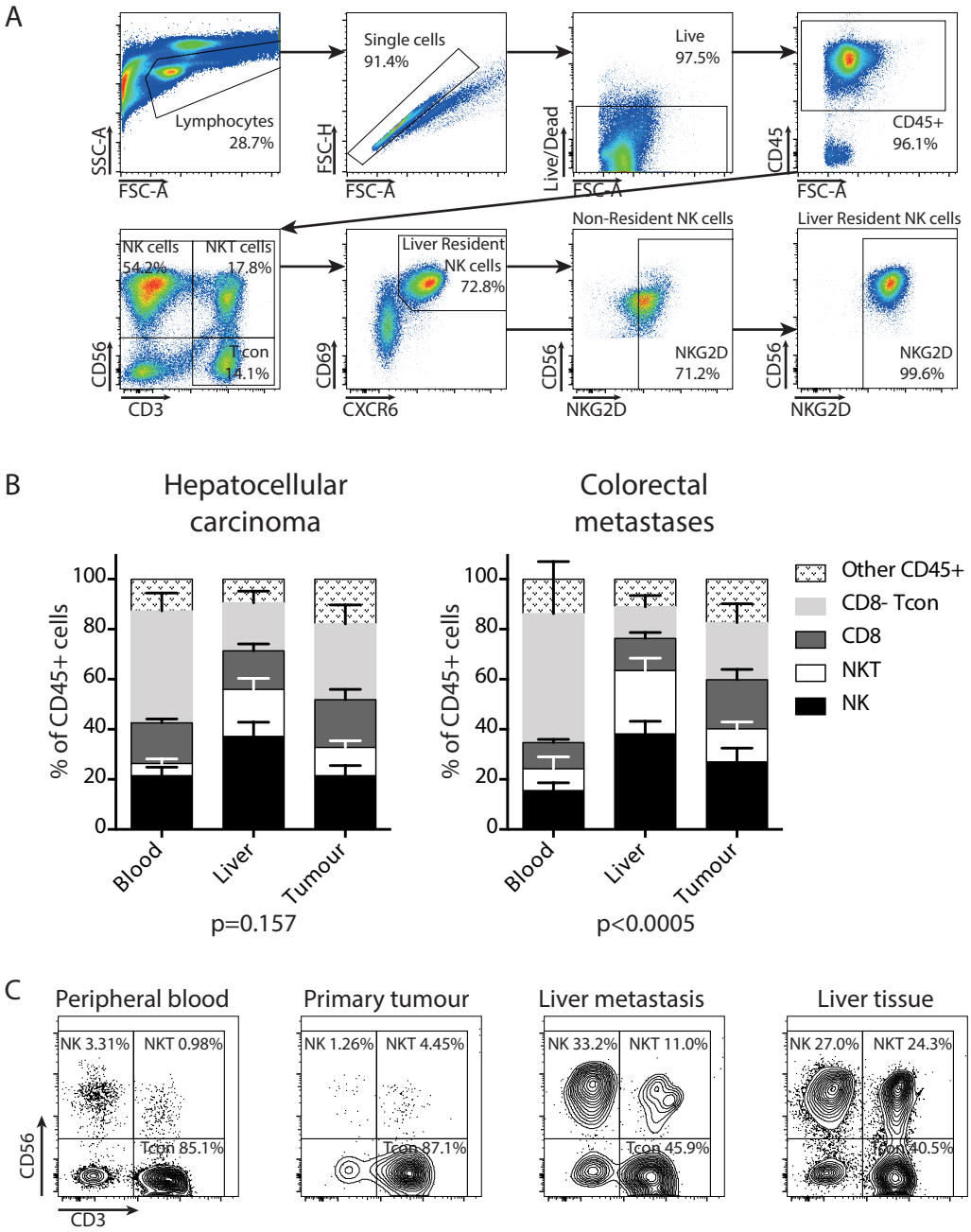

Figure 4.1 Relative proportions of CD45+ cells in peripheral blood, liver tissue and primary and secondary liver tumours

A. Gating strategy for identification of intrahepatic/tumour infiltrating NK cells by flow cytometry, showing identification of CXCR6+ CD69+ liver resident NK cells (CD45+ CD56+ CD3- CD69+ CXCR6+).

B. Proportion of NK cells, NKT cells, CD8+ T cells, CD8- T cells in blood, liver and tumour tissue identified by flow cytometry in HCC (n=6) and CRC (n=10). Bars show mean and SEM, p values determined by MANOVA.

C. Flow cytometry dot plots showing CD45 positive lymphocytes divided into NK, NKT and conventional T cells (Tcon) for lymphocytes derived from peripheral blood, primary colonic adenocarcinoma, liver metastasis and unaffected liver tissue in one individual.

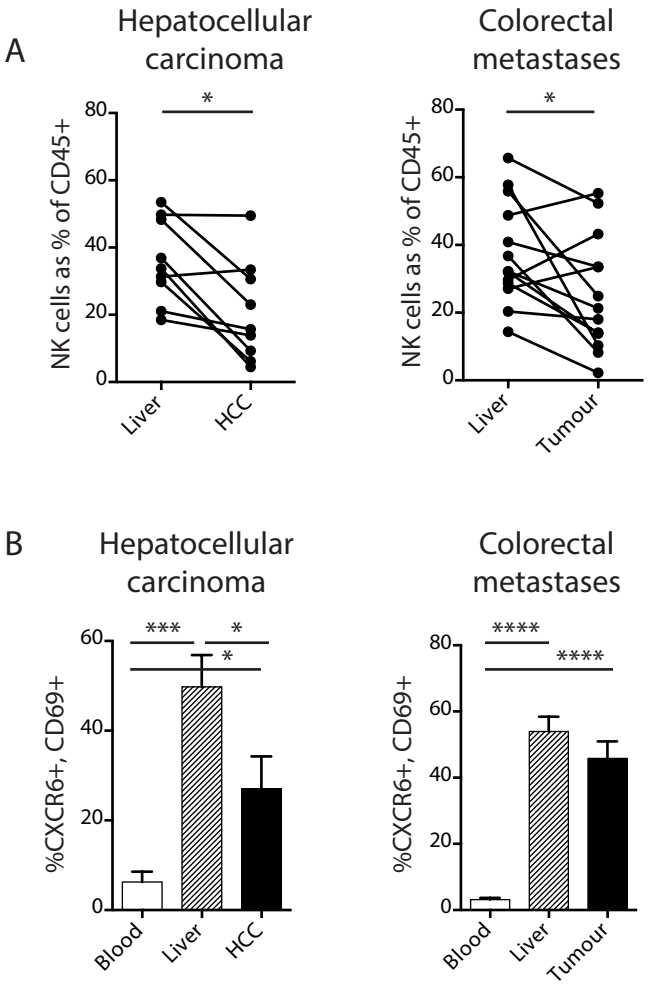

Figure 4.2 Liver resident NK cells infiltrate liver tumours
A. Total NK cells (CD56+ CD3-) as a proportion of CD45+ lymphocytes in HCC (n=9) and CRC (n=13) paired liver and tumour by flow cytometry.
B. CXCR6+ CD69+ NK cells as a proportion of total NK cells in blood, liver and tumour from HCC (n=10) and CRC (n=13) patients by flow cytometry.
Groups were compared using Mann-Whitney U test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

resident population of NK cells that expresses CXCR6 and CD69 and is transcriptionally distinct from their circulating counterparts(Stegmann et al. 2016) (Fig 4.1 A). We therefore asked whether tumour infiltrating NK cells contained any of the liver-resident CXCR6+CD69+ fraction. We found that both HCC and CRC metastases contained a high proportion of CXCR6+CD69+ NK cells compared to their very low frequencies in blood. Thus both HCC and CRC metastases had an NK cell composition that was more reflective of the surrounding liver tissue than the periphery, with a similar proportion of liver resident and liver-infiltrating (CXCR6-CD69-) NK cells to the surrounding healthy liver in colorectal metastases, and in HCC a proportion intermediate between that of blood and liver (Fig 4.2 B).

Next we assessed levels of the major activating receptor NKG2D on tumour-infiltrating NK cells as this is implicated in tumour surveillance. NKG2D ligands are known to be expressed on both HCC and CRC, where their presence is prognostic (Kamimura et al. 2012; McGilvray et al. 2009). Within individuals, NKG2D expression was reduced on tumour-infiltrating NK cells compared with NK cells in unaffected liver in both HCC (Fig 4.3 A) and CRC (Fig 4.3 B). Notably, NKG2D expression is higher on liver resident NK cells in liver tissue and both tumours (Fig 4.3 C). By contrast the expression of NKp46, an activatory receptor not known to be implicated in interactions with these tumours, was not altered on global NK cells from colorectal cancer metastases or primary hepatocellular carcinoma. There was no change in NKp46 expression by either the liver resident or non-resident populations in either tumour (Fig 4.4 A, B, C). There was also no change in the expression of the activation marker HLA-DR *ex vivo* on global NK cells or on either subset (Fig 4.5 A, B). In recent years there has been interest in so-called “adaptive” or “memory-like” NK cells, particularly with regard to intrahepatic NK cells, with this population believed to contain a memory NK pool marked by CXCR6 in some mouse models (Paust et al. 2010). In humans CD57+ NKG2C+ “adaptive” NK cells are most commonly associated with CMV

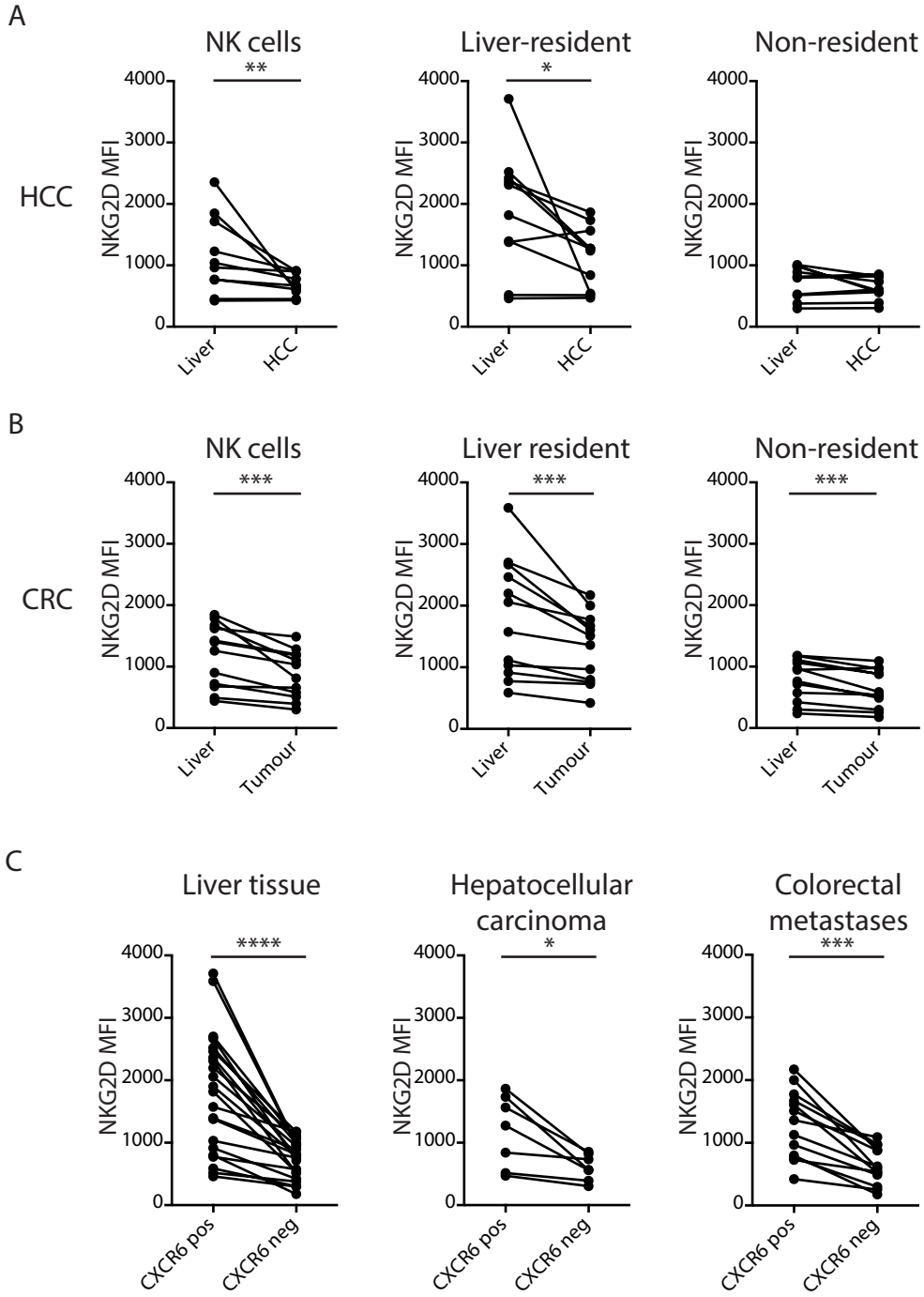

Figure 4.3 Tumour infiltrating NK cells show evidence of NKG2D downregulation
A. NKG2D expression by mean fluorescence intensity (MFI) on total NK cells, liver resident (CXCR6+ CD69+) and non-resident (CXCR6- CD69-) NK cells in HCC and paired liver (n=10) quantified by flow cytometry.
B. NKG2D expression by MFI on total NK cells, liver resident (CXCR6+ CD69+) and non-resident (CXCR6- CD69-) NK cells in CRC and paired liver (n=13) quantified by flow cytometry.
C. NKG2D expression by MFI on liver resident (CXCR6+ CD69+) and non-resident (CXCR6- CD69-) intrahepatic (n=23), HCC infiltrating (n=10) and CRC infiltrating (n=13) NK cells quantified by flow cytometry.
Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

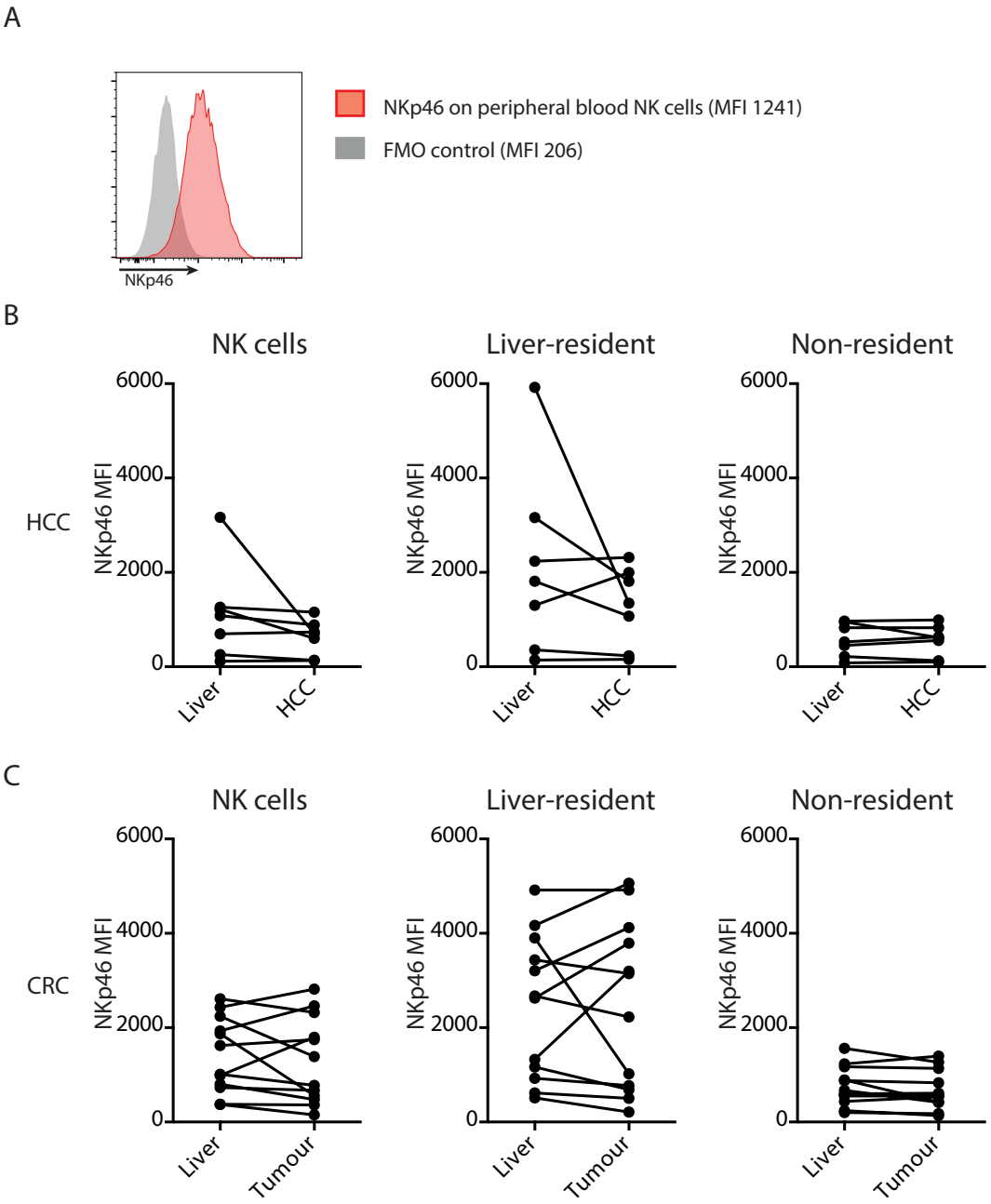

Figure 4.4 NKp46 expression is preserved on tumour-infiltrating NK cells
A. Example of NKp46 staining by flow cytometry, showing fluoresence minus-one (FMO) control and NKp46 stained peripheral blood NK cells. Mean fluorescence intensity (MFI) is shown for both conditions.
B. NKp46 expression as MFI on total NK cells, liver resident (CXCR6+ CD69+) and non-resident (CXCR6- CD69-) NK cells in HCC and paired liver (n=10) quantified by flow cytometry.
C. NKp46 expression as MFI on total NK cells, liver resident (CXCR6+ CD69+) and non-resident (CXCR6- CD69-) NK cells in CRC and paired liver (n=13) quantified by flow cytometry.
Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

(Cerwenka & Lanier 2016), but have also been seen in response to other viral infections such as hantavirus (Björkström et al. 2011), and show strong responses to re-challenge. In both liver tissue and in the tumours, very few CD57+ NKG2C+ were seen, and where paired blood was available the proportion in the liver and tumour were not expanded compared to the peripheral blood (Fig 4.5 C, D).

Having established that NK cells form a large part of the lymphocytic infiltrate in both primary and secondary tumours in the liver, and that they reflect the liver resident and liver-infiltrating population of NK cells, rather than the circulating fraction, we proceeded to consider whether these cells have anti-tumour function. The phenotypes of NK cells infiltrating HCC and CRC were very similar, so these groups have been considered together. We assessed granzyme B and perforin as important cytotoxic mediators in NK cells. Perforin is required to form pores in the lipid bilayer of target cells and granzyme B enters via these pores to cleave caspases and induce apoptosis in the target cell. In line with our previous work, liver resident NK cells were granzyme B-low compared with peripheral blood NK cells or non-resident NK cells found in the liver (Stegmann et al. 2016) (Fig 4.6 A). Granzyme B expression was lower in liver NK cells than in peripheral blood, and there was a trend towards lower granzyme B in tumour NK cells than in peripheral blood NK cells ($p=0.16$ Fig 4.6 B). Granzyme B expression was lowest in liver-resident NK cells infiltrating tumour, suggesting that these cells have either completely degranulated in response to the tumour or are functionally exhausted. This difference was not seen between CXCR6- NK cells in the liver and tumour. This may be because these cells are present in the tissue only transiently before recirculating via the bloodstream. In a separate cohort of patients who underwent liver biopsy for HCC diagnosis, perforin expression was similarly reduced in tumour compared to paired blood, (Fig 4.6 C). In keeping with the previous data, NKG2D expression was reduced in NK cells in HCC this liver biopsy cohort (Fig 4.6 D).

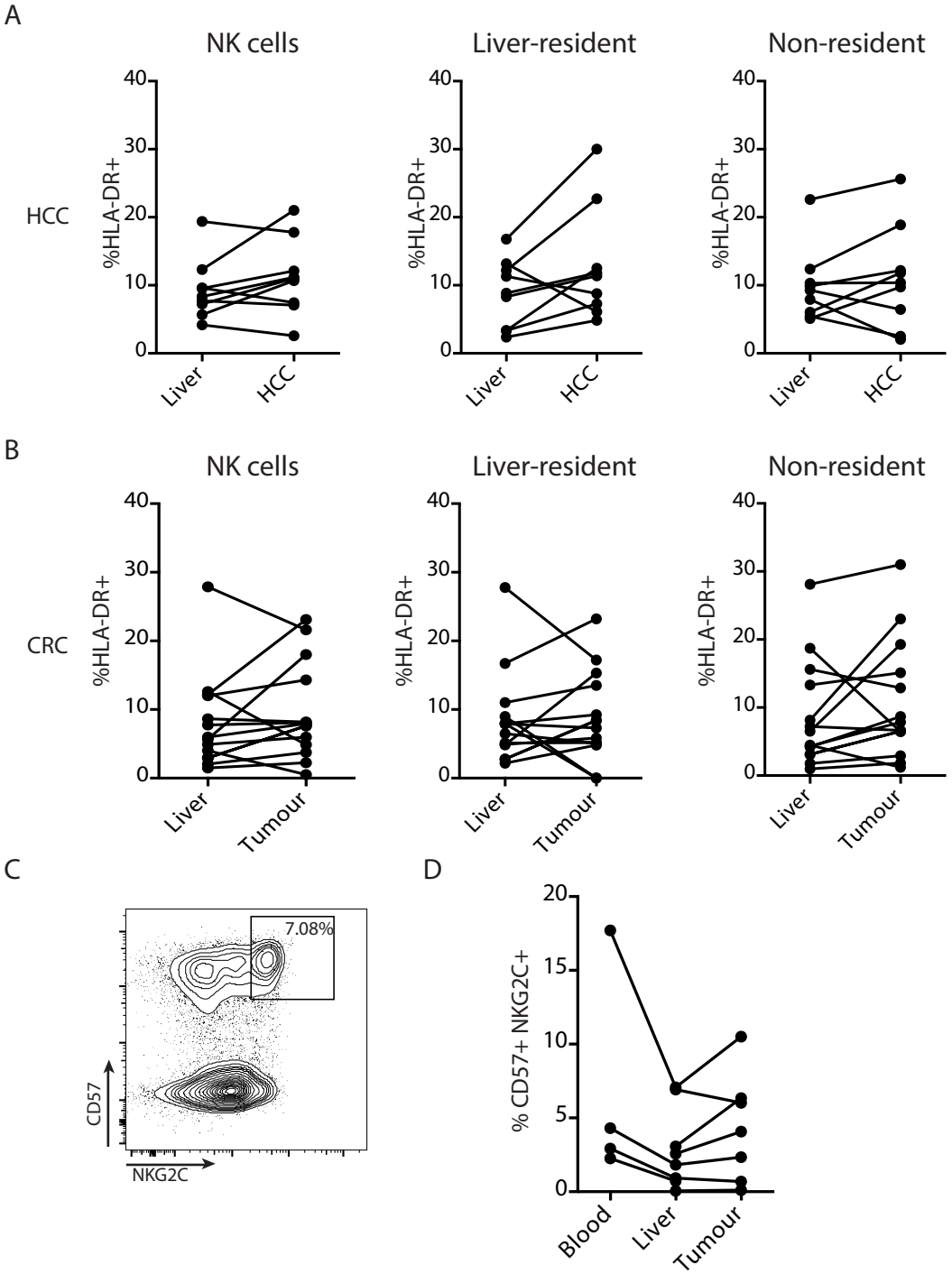

Figure 4.5 No increase in activated or memory-like NK cells infiltrating tumour
A. HLA-DR expression by flow cytometry on total NK cells, liver resident and non-resident NK cells in HCC and paired liver (n=10).
B. HLA-DR expression by flow cytometry on total NK cells, liver resident and non-resident NK cells in CRC and paired liver (n=13).
C. Representative example of flow cytometry do plot showing CD57+ NKG2C+ ex-vivo intrahepatic NK cells.
D. Summary data showing proportion of CD57+ NKG2C+ NK cells in ex-vivo blood (n=4), liver (n=8) and tumour (n=7).
Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

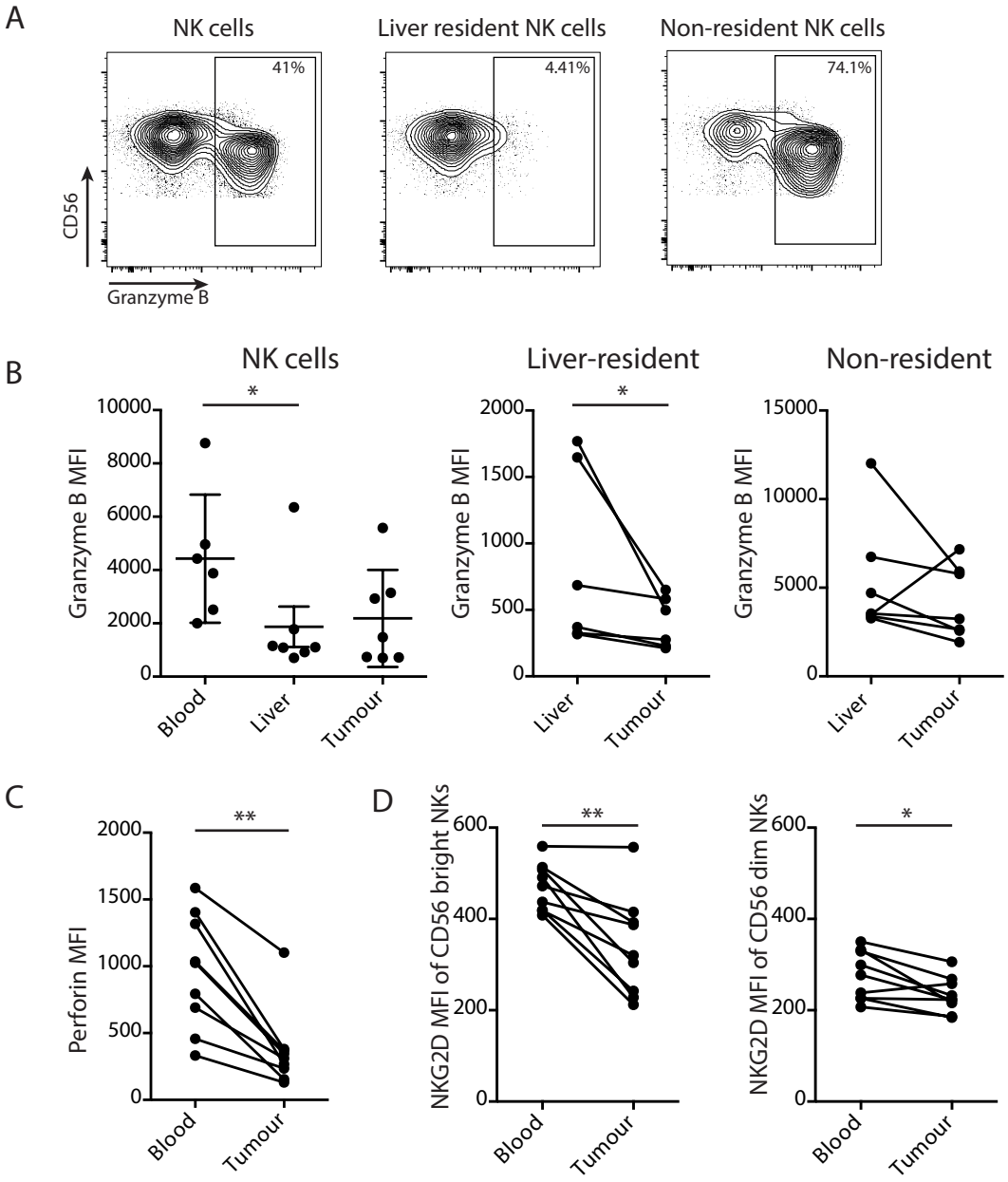

Figure 4.6 Tumour-infiltrating NK cells are granzyme B and perforin-low
A. Representative example of granzyme B staining on intrahepatic NK cells, NK cells, liver resident and non-resident NK cells.
B. Granzyme B expression by flow cytometry, presented as MFI, on total NK cells in peripheral blood, liver and tumour on global NK cells, liver resident and non-resident NK cells.
C. Perforin expression by flow cytometry, presented as MFI, on total NK cells in paired peripheral blood and HCC biopsies on global NK cells.
D. NKG2D expression on CD56 bright and dim NK cells in paired peripheral blood and HCC biopsies (no paired liver tissue was available in these patients), showing that NK cells from tumour biopsies were similar to NK cells from tumour resections.
Groups were compared using Mann-Whitney U test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

The anti-tumour properties of IFNγ are becoming increasingly recognised, and IFNγ knockout mice show accelerated DEN-induced HCC (Meng et al. 2012; Parker et al. 2016). However, IFNγ may also contribute to a carcinogenic inflammatory milieu; in a mouse model of NASH, depletion of IFNγ producing intrahepatic CD4 T cells promoted carcinogenesis(Ma et al. 2016). To assess the capacity of tumour-infiltrating NK cells to produce IFNγ we stimulated PBMC, liver and tumour infiltrating lymphocytes with IL-12 and IL-18 (Fig 4.7 A). Tumour-infiltrating NK cells produced the least IFNγ, in some cases showing a complete inability to respond to stimulation. Peripheral NK cells produced the most IFNγ in all cases (Fig 4.7 B, C). This confirms the impaired cytokine production seen in NK cells in other tumour settings (Platonova et al. 2011; Mamessier et al. 2011). There was very little difference in IFNγ production between liver resident and non-resident NK cells in both liver and tumour, with the inactivity in tumour infiltrating NK cells reflected in both populations (Fig 4.7 D). This may suggest that loss of activity is rapid and persists even after cells have been removed from the tumour microenvironment.

We sought to understand whether the NK cell population inside the tumour was able to maintain itself or whether it requires a constant influx of NK cells from the surrounding liver tissue. To assess whether proliferation was impaired as well as function, tumour and liver lymphocytes were stained for the marker of proliferation, Ki67. Despite the reduced NK cell populations seen infiltrating tumours compared to in liver tissue, tumour-infiltrating NK cells express Ki67 at a similar frequency to their liver counterparts (Fig 4.8 A, B). Among non-resident NK cells within tumours there was a slight increase in Ki67 expression compared with the liver (Fig 4.8 C), possibly indicating an initial proliferation on first encountering tumour cells. This suggests that tumour-infiltrating NK cells are, at least partially, able to maintain themselves in the tumour environment, despite other data revealing impaired activation

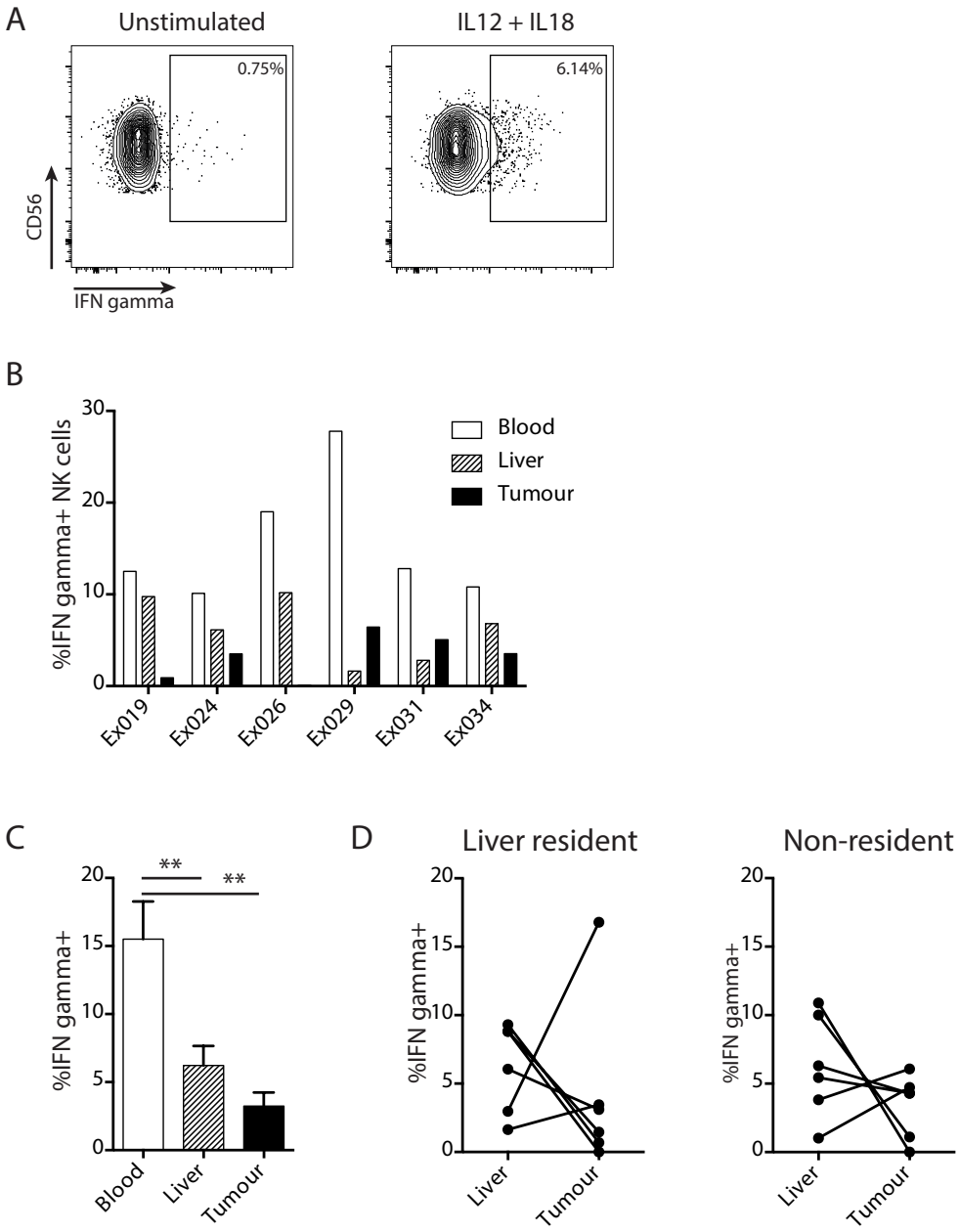

Figure 4.7 Intrahepatic and tumour-infiltrating NK cells are poor producers of interferon gamma

A. Representative example of intracellular cytokine staining for IFNγ by flow cytometry by total intrahepatic NK cells on stimulation with IL12 and IL18.

B. Summary data showing intracellular IFNγ staining by flow cytometry by total NK cells from blood, liver and tumour, grouped by individual.

C. Summary data showing intracellular IFNγ staining by flow cytometry by total NK cells from paired blood, liver and tumour. Bars shown mean and SEM.

D. Intracellular IFNγ staining by flow cytometry by liver resident and non-resident NK cells from paired liver and tumour.

Groups were compared using Mann-Whitney U test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

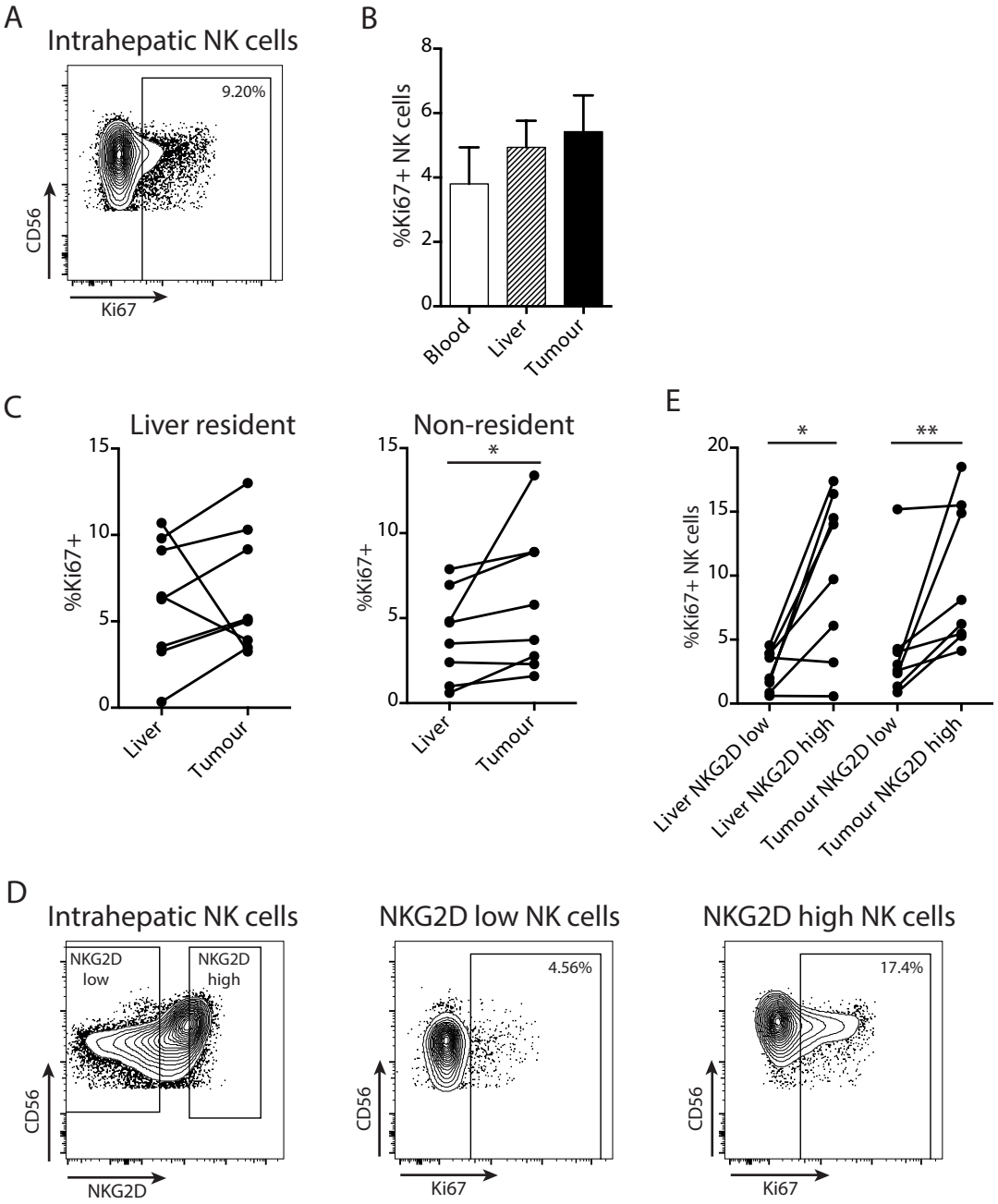

Figure 4.8 Proliferation is maintained in tumour-infiltrating NK cells

A. Representative example of intranuclear Ki67 staining on intrahepatic NK cells by flow cytometry.

B. Summary data for Ki67 expression by flow cytometry on total NK cells from paired blood, liver and tumour. Bars shown mean and SEM.

C. Ki67 expression by liver resident and non-resident NK cells from paired liver and tumour (n=8).

D. Example of gating of highest and lowest 25% of NK cells by NKG2D expression, and Ki67 expression in these two groups.

E. Ki67 expression on the highest and lowest 25% of NK cells by NKG2D expression in liver and tumour (n=8).

Groups were compared using Mann-Whitney U test (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

and function.

NKG2D ligands are found on both HCC and CRC, and NKG2D engagement on NK cells leads to NKG2D internalisation (Quatrini et al. 2015). To examine whether recent NK cell interactions with tumour cells might affect proliferation, Ki67 expression was compared on NKG2D-high NKG2D-low NK cells (gated on the highest and lowest 25% of NKG2D expression respectively). Ki67 expression was higher on the NKG2D-high NK cells than on the NKG2D-low NK cells. This suggests that NKG2D engagement, resulting in NKG2D internalisation, may impair NK cell proliferation in the liver (Fig 4.8 D, E).

4.3.2 Functional inactivation of NK cells can be recapitulated by *in vitro* exposure to HCC

To mimic the interaction between NK cells and liver tumours we used PLC/PRF/5 cells, an adherent line derived from HCC that expresses multiple ligands for NK cell receptors, including HLA class I and the NKG2D ligand MICA (Fig 4.9 A). Overall tumour-infiltrating NK cells resembled liver NK cells much more closely than peripheral NK cells, so NK cells isolated from liver tissue were used to examine the functional impairment in tumour-infiltrating NK cells. Magnetic beads were used to isolate NK cells by negative selection. Intrahepatic NK cells downregulated NKG2D on their cell surface on co-culture with PLC/PRF/5 (Fig 4.10 A,), an effect not seen consistently with peripheral NK cells. This downregulation was seen on both resident and non-resident NK cells (Fig 4.10 B). NK cells, either intrahepatic or from peripheral blood, co-cultured with HCC cells overnight do not upregulate the activation markers HLA-DR or CD25 (Fig 4.11 A, B). Instead there is a small but statistically significant reduction in HLA DR expression on intrahepatic NK cells following coculture. PLC/PRF/5 cells did not induce NK cells to degranulate on coculture (Fig 4.11 C). Similar to our *ex vivo* observations,

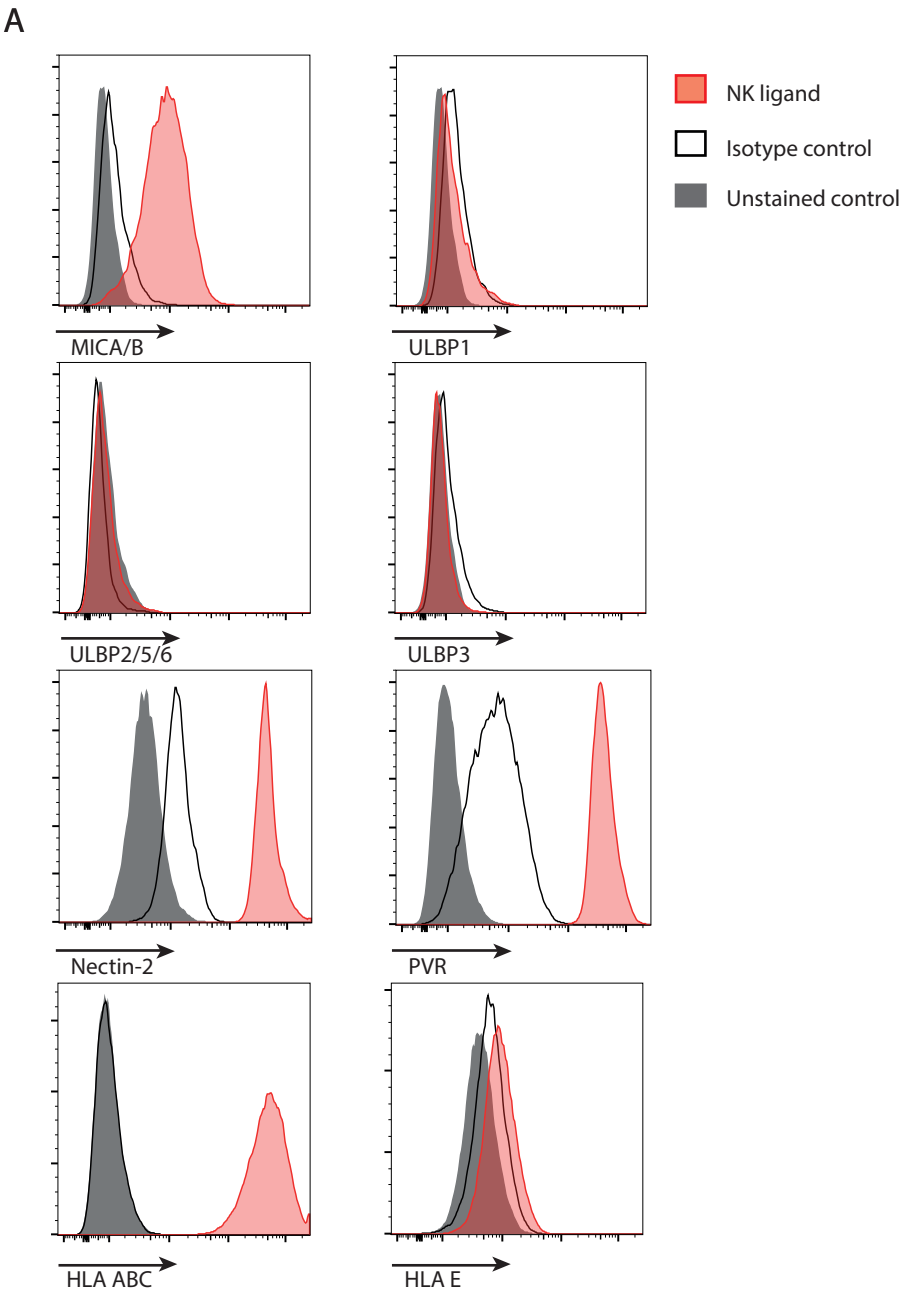

Figure 4.9 PLC/PRF/5 cells express ligands for NK cell receptors
A. Flow cytometric assessment of the expression of a range of ligands for NK cell receptors by PLC/PRF/5 cells. Expression of ligands for NKG2D (MICA/B, ULBP1-3), DNAM1 (Nectin-2, PVR), KIR (HLA A, B, C) and NKG2A/C (HLA E) shown as histograms. Representative example of 3 independent experiments.

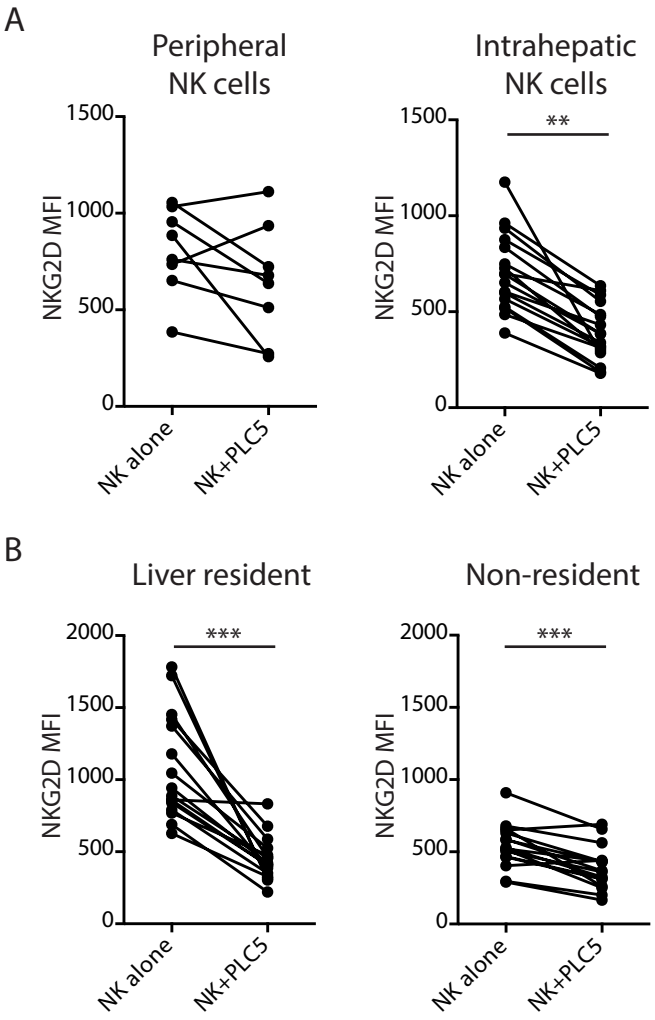

Figure 4.10 PLC/PRF/5 cells induce NKG2D downregulation on intrahepatic NK cells

A. NKG2D expression by flow cytometry and expressed as MFI by peripheral (n=8) and intrahepatic (n=17) NK cells following 18 hour co-culture with PLC/PRF/5 cells.

B. NKG2D expression by flow cytometry and expressed as MFI by liver resident and non-resident intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells (n=16). PLC5, PLC/PRF/5 (Alexander) cells.

Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

NKp46 is maintained on coculture (Fig 4.11 D), and there is a small but consistent reduction in the proportion of liver resident NK cells recovered after coculture with HCC cells (Fig 4.11 E). NKG2D downregulation does not occur when NK cells are cultured in 50% PLC/PRF/5 culture supernatant or when NK cells and PLC/PRF/5 are separated by a transwell insert (Fig 4.12 A), confirming the requirement for contact. This implies that NK cells are interacting with PLC/PRF/5 HCC cells via NKG2D-NKG2DL, a process which would be expected to lead to NKG2D internalisation and subsequent signalling via the ERK and AKT pathways. To investigate the fate of cell-surface NKG2D we used imaging cytometry to visualise the cellular localisation of NKG2D molecules. NK cells co-cultured with PLC/PRF/5 overnight internalise NKG2D (Fig 4.12 B, C), which accounts for the decreased cell surface expression. NKG2D internalisation is usually associated with ERK phosphorylation. In one example using intrahepatic NK cells, PLC/PRF/5 contact resulted in ERK activation that was greater than that induced by IL-15, which also signals via ERK (Horng et al. 2007; Jabri & Abadie 2015)(Fig 4.13 A, B). This implies another interaction at the immunological synapse or as a result of close contact with PLC/PRF/5 HCC cells, is impeding NK cell activation despite NKG2D signalling, and thereby preventing activation and cytotoxicity.

In order to quantify changes to NKG2D cytotoxic function after contact with liver tumour, we used a two-stage challenge model (Fig 4.14 A). K562 cells do not express HLA class-I molecules and are spontaneously lysed by NK cells (Langhans et al. 2005). They express the NKG2D ligands MICA, MICB, ULBP1 and ULBP2 as well the adhesion molecule ICAM-1 (Boissel et al. 2006). They may express low levels of ligands for NKp30 and NKp44 but blocking antibodies against these receptors, and against NKp46, do not affect K562 killing by NK cells (Byrd et al. 2007), whereas NKG2D blocking markedly impairs NK cell cytotoxicity against K562 cells, and in combination with anti-LFA-1 (the receptor for ICAM-1) blocking cytotoxicity is almost

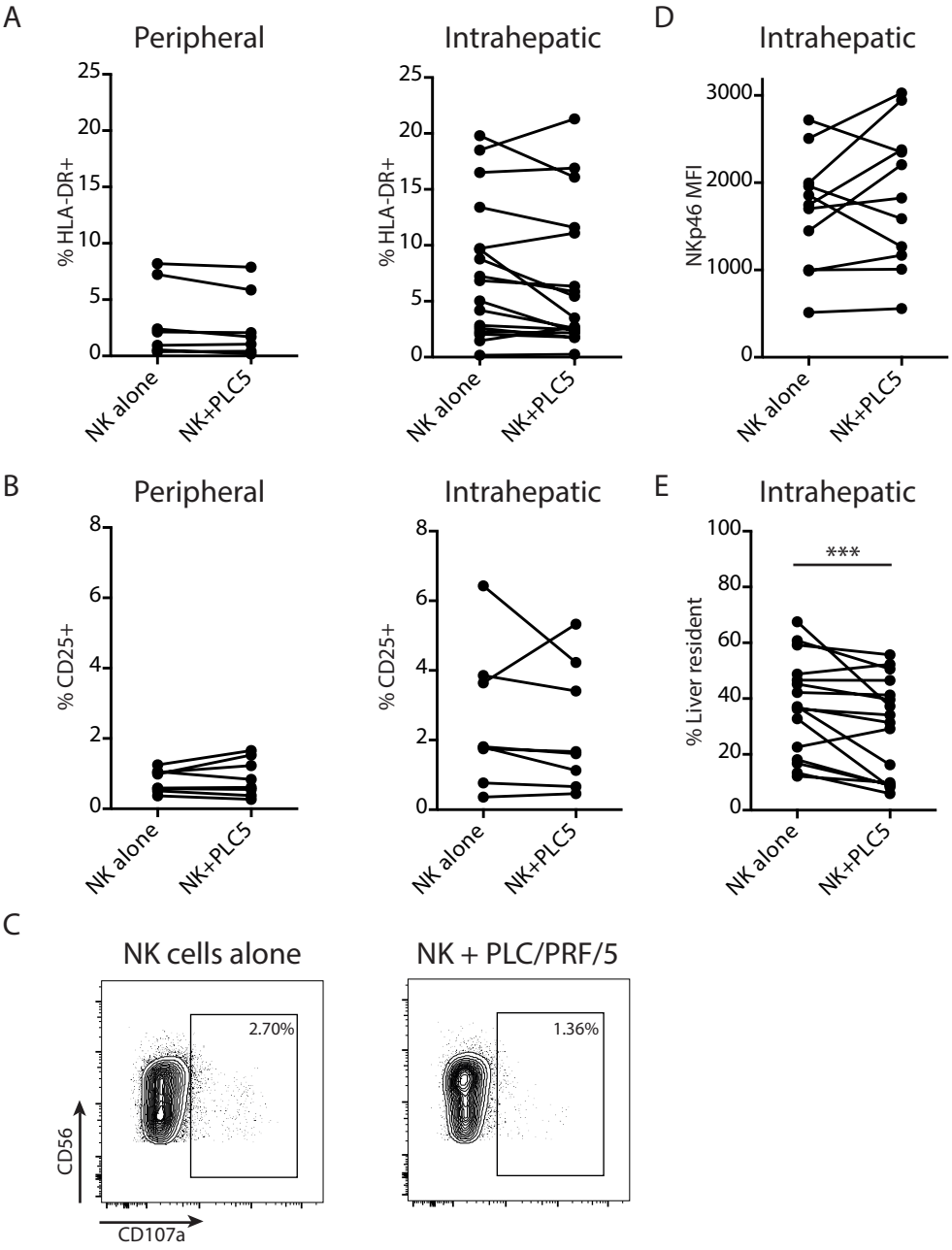

Figure 4.11 Coculture with PLC/PRF/5 cells does not activate NK cells

A. HLA DR expression by flow cytometry on peripheral and intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells (n=17).

B. CD25 expression by flow cytometry on peripheral and intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells (n=10).

C. Representative example of CD107a staining by flow cytometry on intrahepatic NK cells following 18 hour coculture with PLC/PRF/5 cells.

D. NKp46 expression by flow cytometry, expressed as MFI, on intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells (n=11).

E. Liver resident CXCR6+ CD69+ cells, identified by flow cytometry, as a percentage of total NK cells following 18 hour co-culture with PLC/PRF/5 cells (n=16).

Groups were compared using Wilcoxon matched-pairs signed rank test. $P \leq 0.05$ was considered to be significant for all tests. Figures are labelled: *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

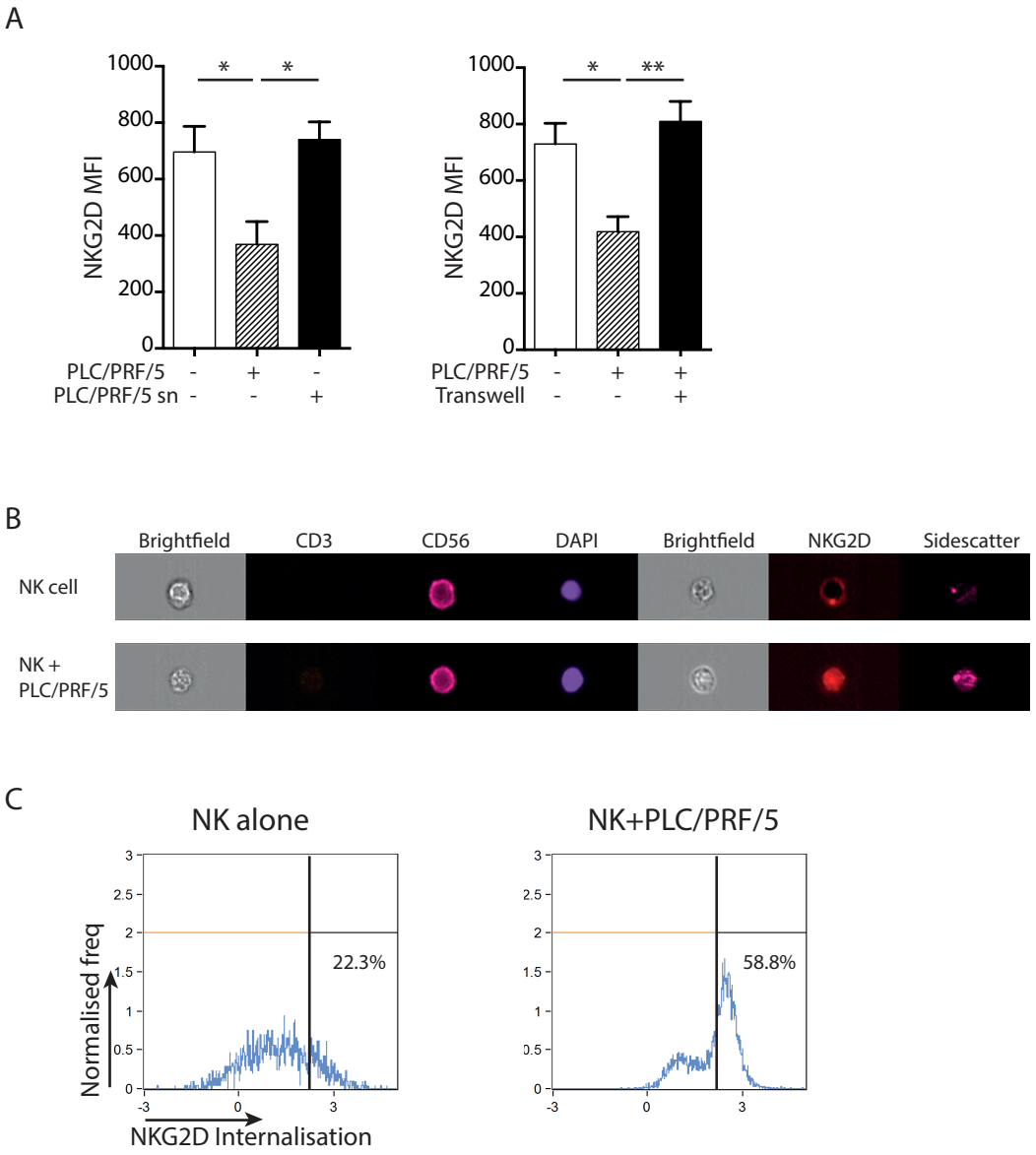

Figure 4.12 NKG2D is internalised on contact with PLC/PRF/5 cells
A. NKG2D expression by intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells separated by transwell (n=4) and with culture supernatant diluted 1:1 with fresh media (n=4). Groups compared by unpaired t-test with Welch's correction.
B. Representative example showing NKG2D internalisation on NK cells following overnight coculture with PLC/PRF/5 cells by imaging cytometry.
C. Summary data showing internalisation of NKG2D following overnight coculture with PLC/PRF/5 cells. Internalisation score as computed by IDEAS software as described in Materials and Methods.
sn, supernatant.
P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤ 0.005; ***, p ≤0.001; ****, p ≤0.0001.

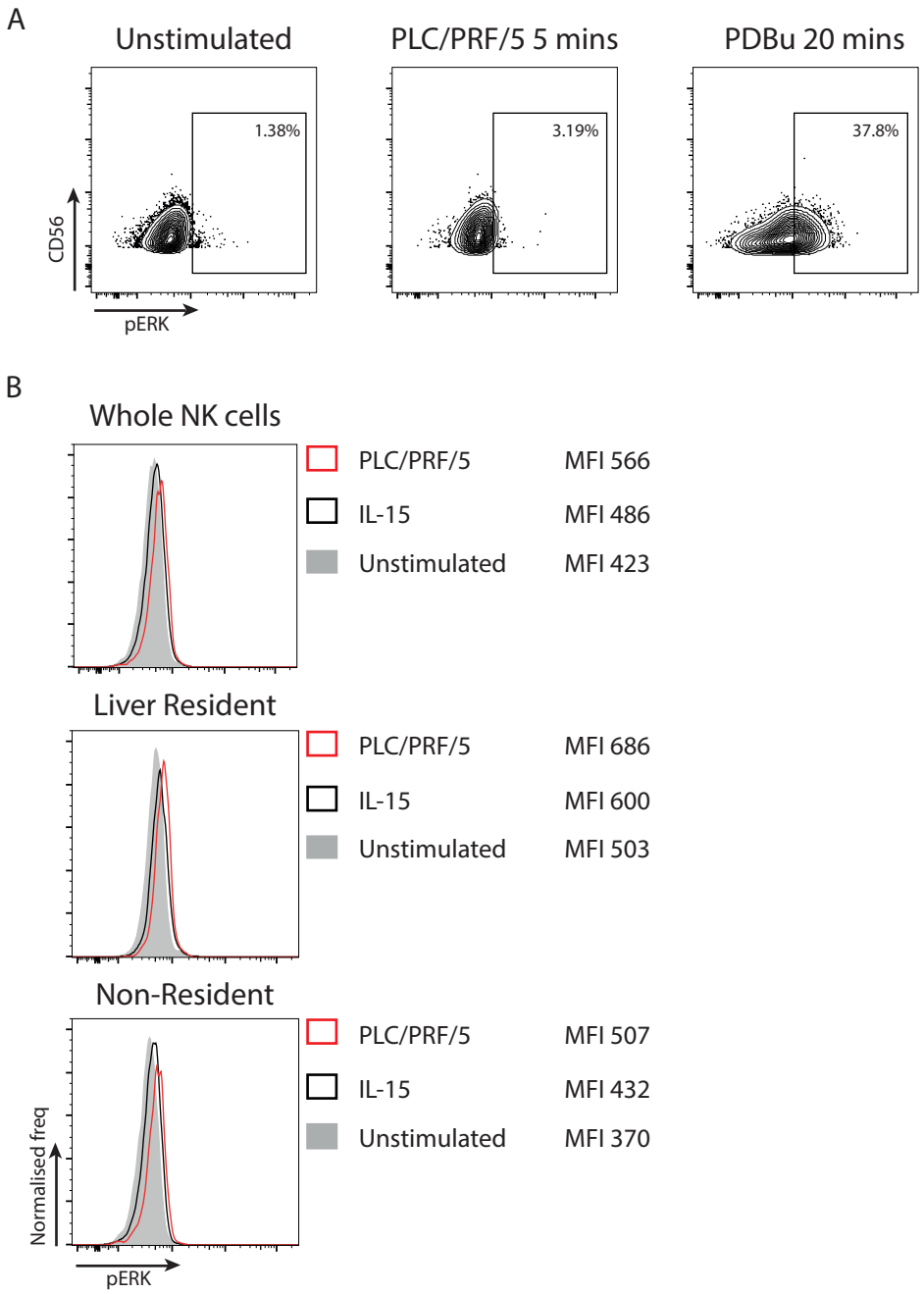

Figure 4.13 Coculture with PLC/PRF/5 cells induces ERK phosphorylation in NK cells
A. Representative example of flow cytometric staining of phospho-ERK in intrahepatic NK cells. Whole intrahepatic lymphocytes were incubated at 37°C with phorbol 12, 13 dibutyrate (PDBu) for 20 mins as a positive control or with PLC/PRF/5 cells for 5 mins or were unstimulated.
B. Comparison of pERK staining by flow cytometry after 5 mins PLC/PRF/5 stimulation, 5 mins 50ng/ml IL-15 stimulation and unstimulated whole intrahepatic, liver resident and non-resident intrahepatic NK cells. Phospho-ERK MFIs are shown for each condition.
With thanks to Leo Swadling

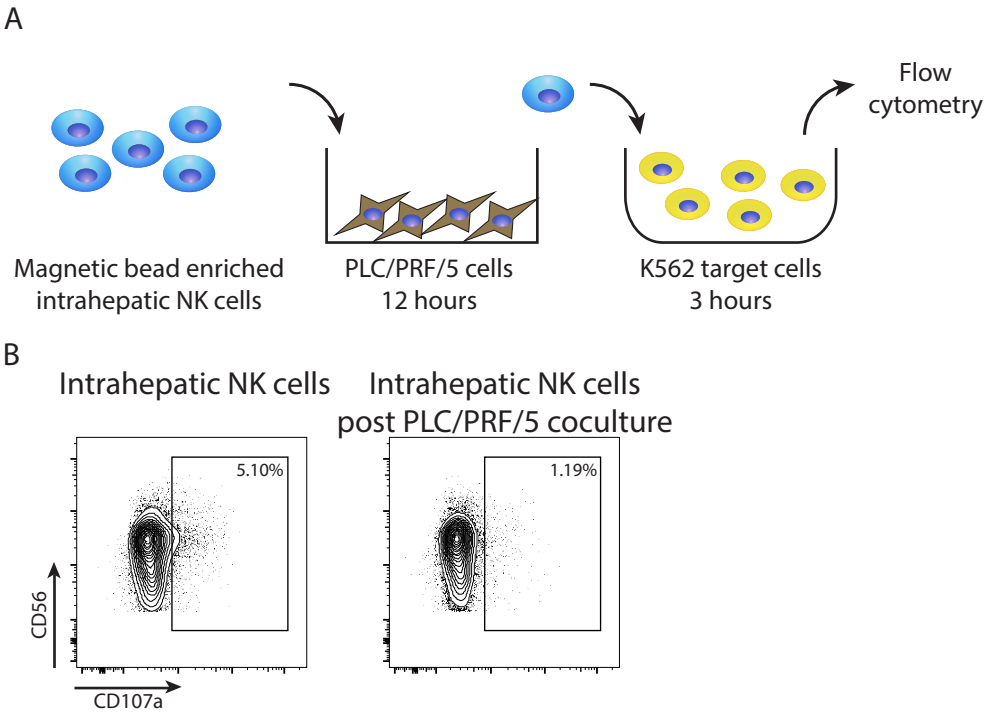

Figure 4.14 Experimental design to quantify NK cell cytotoxic potential after exposure to PLC/PRF/5 cells

A. Diagram showing experimental design of PLC/PRF/5 coculture followed by K562 challenge.

B. Representative example of CD107a staining measured by flow cytometry on intrahepatic NK cells following 18 hour coculture with PLC/PRF/5 cells followed by challenge with K562 cells.

completely abrogated (X. Chen et al. 2007). After co-culture with PLC/PRF/5 cells overnight, NK cells were washed off and challenged with K562 cells at 1:1 ratio to assess ongoing cytotoxicity. Cytotoxic potential, as measured by CD107a expression, was consistently reduced following PLC/PRF/5 co-culture in both peripheral and intrahepatic NK cells (Fig 4.14 B, 4.15 A). This effect is seen consistently in both resident and non-resident NK cell populations (Fig 4.15 B), although liver-resident NK cells have a lower level of baseline degranulation. IFNγ production is also significantly impaired after coculture with PLC/PRF/5 HCC cells (Fig 4.16 A), with the bulk of the decline seen in non-resident NK cells (Fig 4.16 B). There was a reduction in activation, measured by HLA-DR, on K562 challenge after PLC/PRF/5 coculture (Fig 4.16 C), in keeping with an inhibitory effect of the HCC line. CD25 expression was unchanged (Fig 4.16 D). Similar impairment of degranulation was seen when isolated intrahepatic NK cells, stained with cell trace violet to distinguish them from tumour infiltrating NK cells, were cocultured overnight with a 5mm diameter slice of autologous HCC (Fig 4.17 A). This suggests that the NK cell functional impairment caused by PLC/PRF/5 coculture is not caused by KIR-HLA mismatch or some other allogeneic effect but is a property of HCC itself.

The reduction in degranulation is dependent on contact (Fig 4.18 A) but is not recovered by the blockade of NKG2D and DNAM1, either individually or in combination, suggesting NKG2D engagement is not the dominant mechanism by which cytotoxicity is impaired (Fig 4.18 B). Soluble NKG2D also did not prevent NKG2D downregulation on NK cells (Fig 4.18 C). Blockade of NKG2D using recombinant human ULBP1 or anti-MICA also had no effect on function (Fig 4.18 D).

Cytotoxic dysfunction is maintained in the face of prevention of KIR engagement using HLA-ABC blocking antibodies (Fig 4.19 A). Similarly,

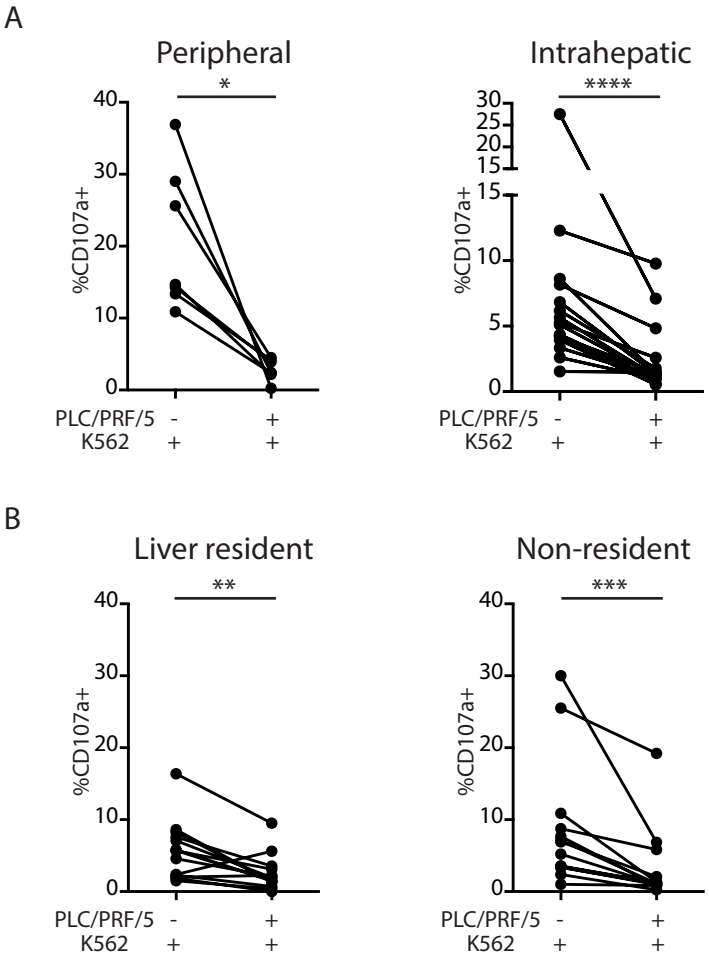

Figure 4.15 NK cell cytotoxicity is impaired following coculture with PLC/PRF/5 cells
A. Summary data showing CD107a expression measured by flow cytometry following K562 challenge after PLC/PRF/5 coculture on peripheral (n=7) and intrahepatic NK cells (n=16).
B. Summary data showing CD107a expression measured by flow cytometry following K562 challenge after PLC/PRF/5 coculture on liver resident and non-resident intrahepatic NK cells (n=14).
Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

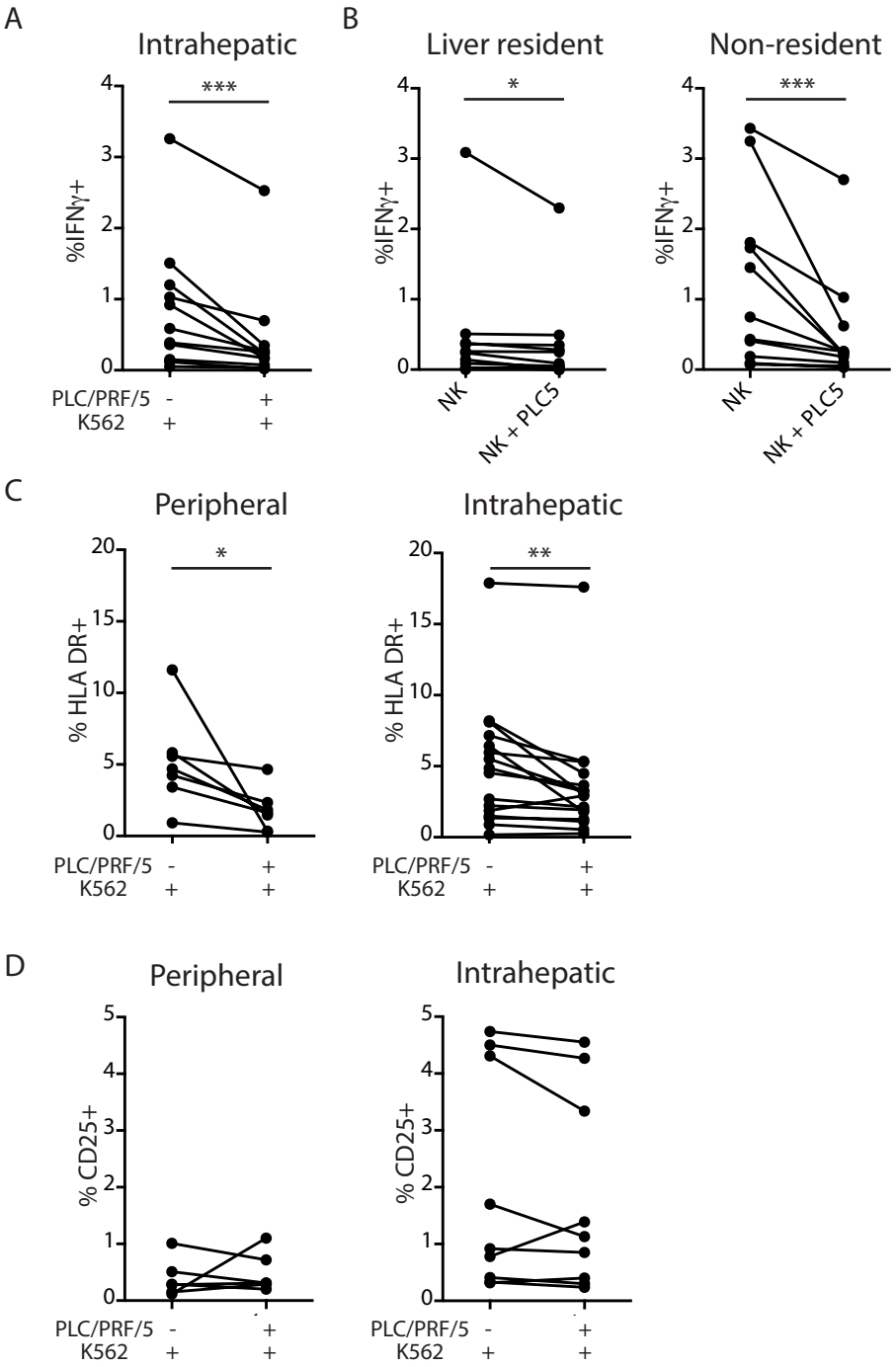

Figure 4.16 PLC/PRF/5 cells impair NK cell cytokine production

A. Intracellular IFNγ staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture in intrahepatic NK cells (n=11).

B. Intracellular IFNγ staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture in liver resident and non-resident intrahepatic NK cells (n=11).

C. HLA DR expression by flow cytometry on peripheral (n=7) and intrahepatic (n=16) NK cells following K562 challenge after PLC/PRF/5 coculture.

D. CD25 expression by flow cytometry on peripheral (n=6) and intrahepatic (n=9) NK cells following K562 challenge after PLC/PRF/5 coculture.

Groups were compared using Wilcoxon matched-pairs signed rank test. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤ 0.001; ****, p ≤0.0001.

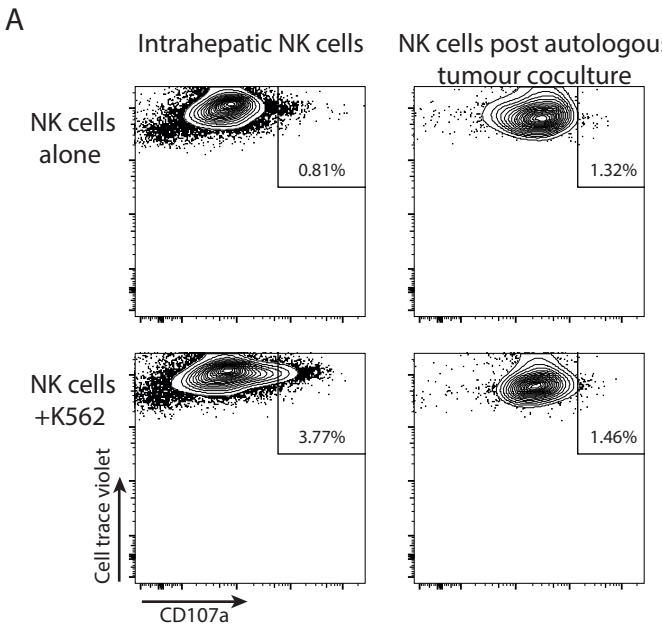

Figure 4.17 NK cell cytotoxicity is impaired following coculture with autologous tumour

A. Representative example of CD107a expression by flow cytometry on intrahepatic NK cells following 18 hour coculture with autologous HCC followed by challenge with K562 cells. Cells were gated on NK cells then cell trace violet positive to distinguish added intrahepatic NK cells from tumour-infiltrating NK cells present in the autologous tumour.

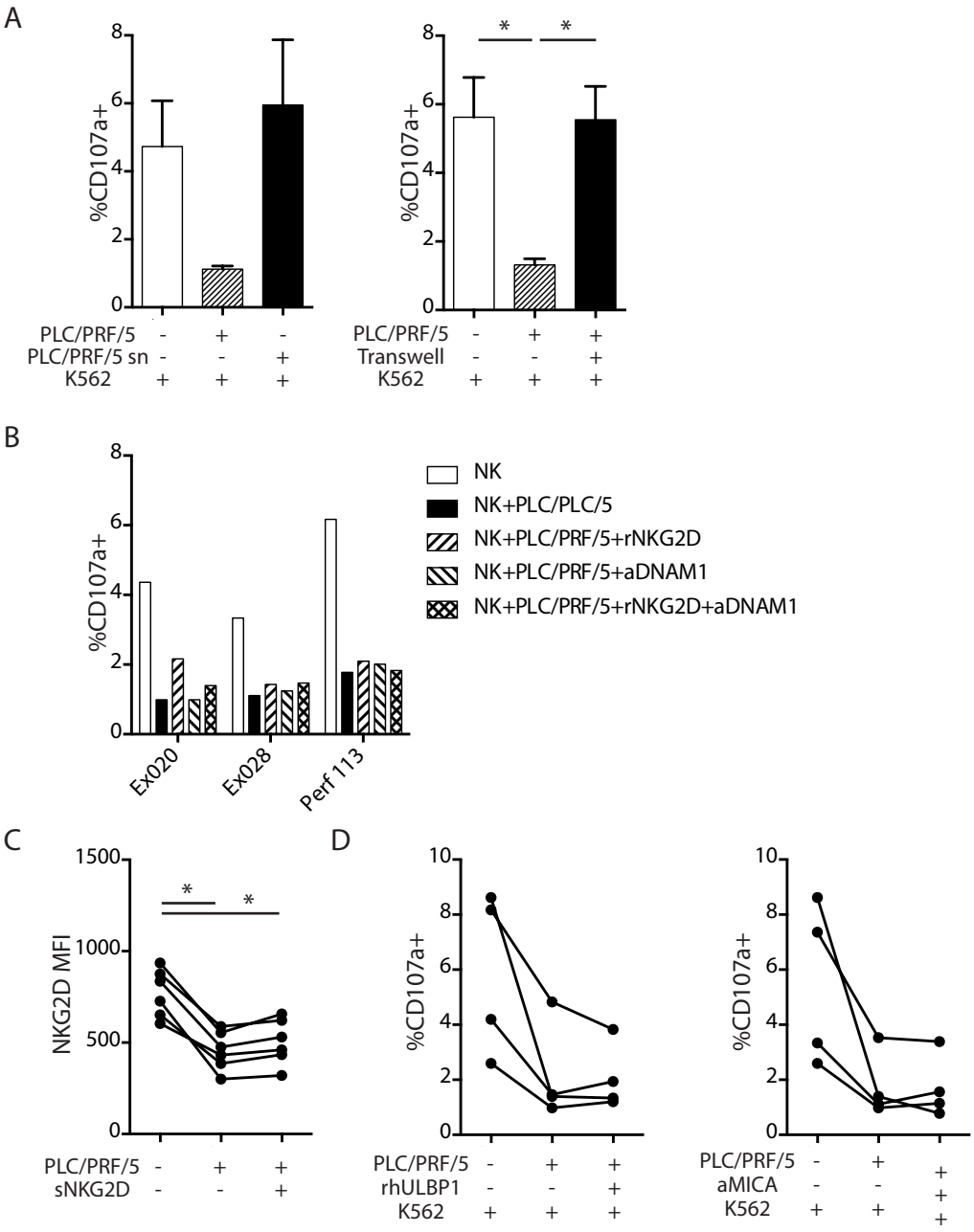

Figure 4.18 NKG2D blockade does not prevent PLC/PRF/5 mediated NK cell functional inhibition

A. CD107a staining by flow cytometry following K562 challenge after co-culture with PLC/PRF/5 cells separated by transwell and with culture supernatant diluted 1:1 with fresh media.

B. CD107a staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture with NKG2D and DNAM-1 blockade. Ex, explant; Perf, perfusate.

C. NKG2D expression by flow cytometry of peripheral and intrahepatic NK cells following 18 hour co-culture with PLC/PRF/5 cells with and without soluble NKG2D.

D. CD107a staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture with recombinant human ULBP1 and anti-MICA blocking antibody.

Groups were compared using unpaired t test with Welch's correction (unpaired) and Wilcoxon matched-pairs signed rank test (paired analyses). P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001; ****, p ≤0.0001.

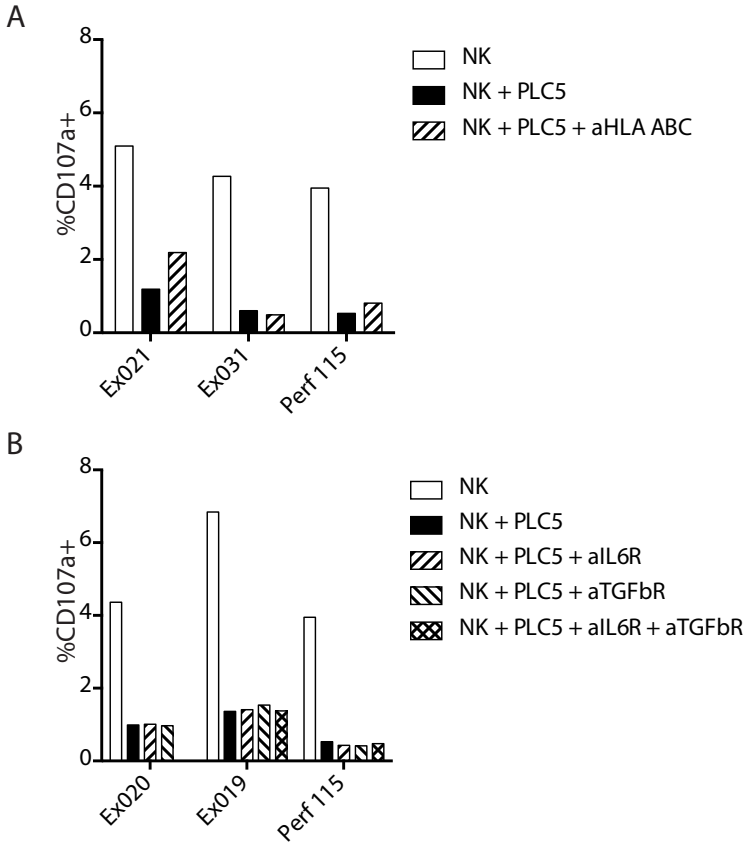

Figure 4.19 PLC/PRF/5 mediated NK cell functional inhibition is unaffected by HLA-ABC, IL6 or TGFβ blockade

A. CD107a staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture with anti-HLA class I blocking antibody.

B. CD107a staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture with anti-TGFβ receptor and anti-IL6 receptor blocking antibodies.

aHLA ABC, anti-HLA-A, B, C blocking antibody; aIL6R, anti-IL-6 receptor alpha blocking antibody; aTGFbR, anti-TGFβ receptor blocking antibody.

blockade of TGFβ and IL-6 signalling; the former important in liver fibrosis, the latter an important driver of liver carcinogenesis and associated with impaired NK cell function; had no effect on CD107a expression after K562 challenge (Fig 4.19 B).

4.3.3 NK cell function can be restored by IL-15

We sought to investigate mechanisms by which NK cell function might be restored. NK cells were removed from overnight PLC/PRF/5 coculture and rested in fresh medium overnight in the presence of different cytokines followed by challenge with K562 cells as before (Fig 4.20 A). Degranulation

was improved by overnight treatment with IL-15 but not IL-2, IL-12 or IL-18 before K562 challenge (Fig 4.20 B). In the same experiments IFNγ production was recovered by all the cytokines tested, but IL-15 showed the most pronounced recovery (Fig 4.20 C). IL-15 consistently restored degranulation and IFNγ production after PLC/PRF/5 coculture (Fig 4.21 A, B). In a stepwise comparison of NK cell function at baseline, after PLC/PRF/5 coculture, after rest and after rest with IL-15, incubation overnight in fresh media alone did not restore NK cell function unless IL-15 was added (Fig 4.21 C, D). IL-15 had only mild effects on NKG2D expression, instead functional recovery seemed to be associated in some cases with an increase in granzyme B (Fig 4.22 A, B).

Added directly into coculture with intrahepatic NK cells and PLC/PRF/5 HCC cells, IL-15 did not induce degranulation (Fig 4.23 A). However IL-15 did have the capacity to recover the function of *ex vivo* tumour-infiltrating NK cells. Intrahepatic and tumour infiltrating lymphocytes from the same liver were incubated with IL-15 or media alone overnight and then challenged with K562. Unlike the intrahepatic NK cells, tumour-infiltrating NK cells were unable to respond to K562 cells unless they had been pre-stimulated with IL-15 (Fig 4.23 B). IL-15 boosted degranulation of tumour-infiltrating NK cells to

higher than the basal level seen upon K562 challenge of NK cells from surrounding liver, although not as high as their IL-15 boosted response.

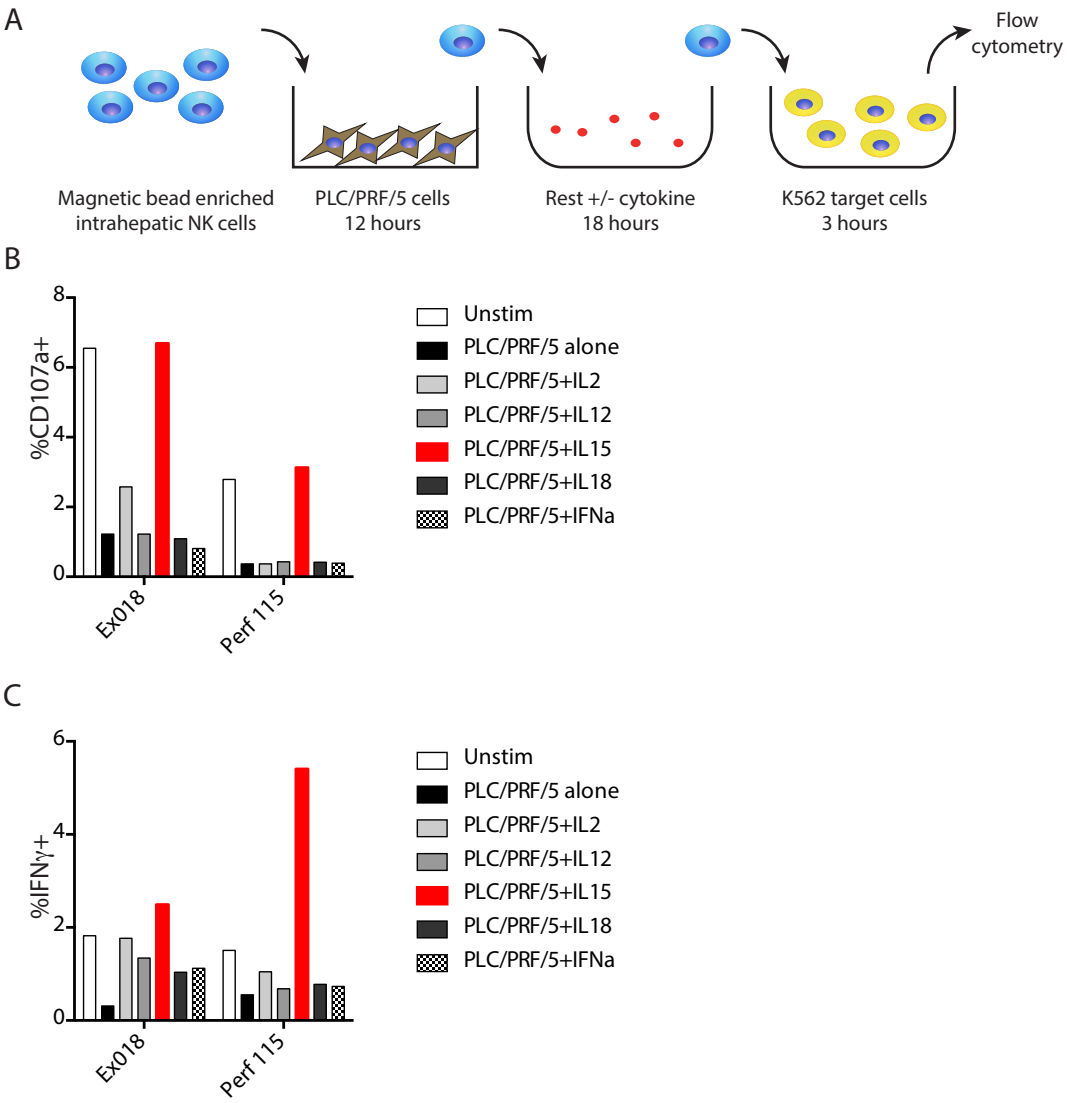

Figure 4.20 IL-15 restores NK cell function following PLC/PRF/5 coculture

A. Diagram showing experimental design of PLC/PRF/5 coculture followed by rest in fresh media and cytokine before K562 challenge.

B. CD107a staining by flow cytometry following K562 challenge after PLC/PRF/5 coculture then rest overnight in fresh media with the appropriate cytokine on intrahepatic NK cells from two individuals, one with NK cells isolated from a liver explant (Ex) and one with NK cells isolated from a liver perfusate (Perf).

C. Intracellular IFNγ staining by flow cytometry in intrahepatic NK cells following K562 challenge after PLC/PRF/5 coculture then rest overnight in fresh media with the appropriate cytokine from two individuals, one with NK cells isolated from a liver explant (Ex) and one with NK cells isolated from a liver perfusate (Perf).

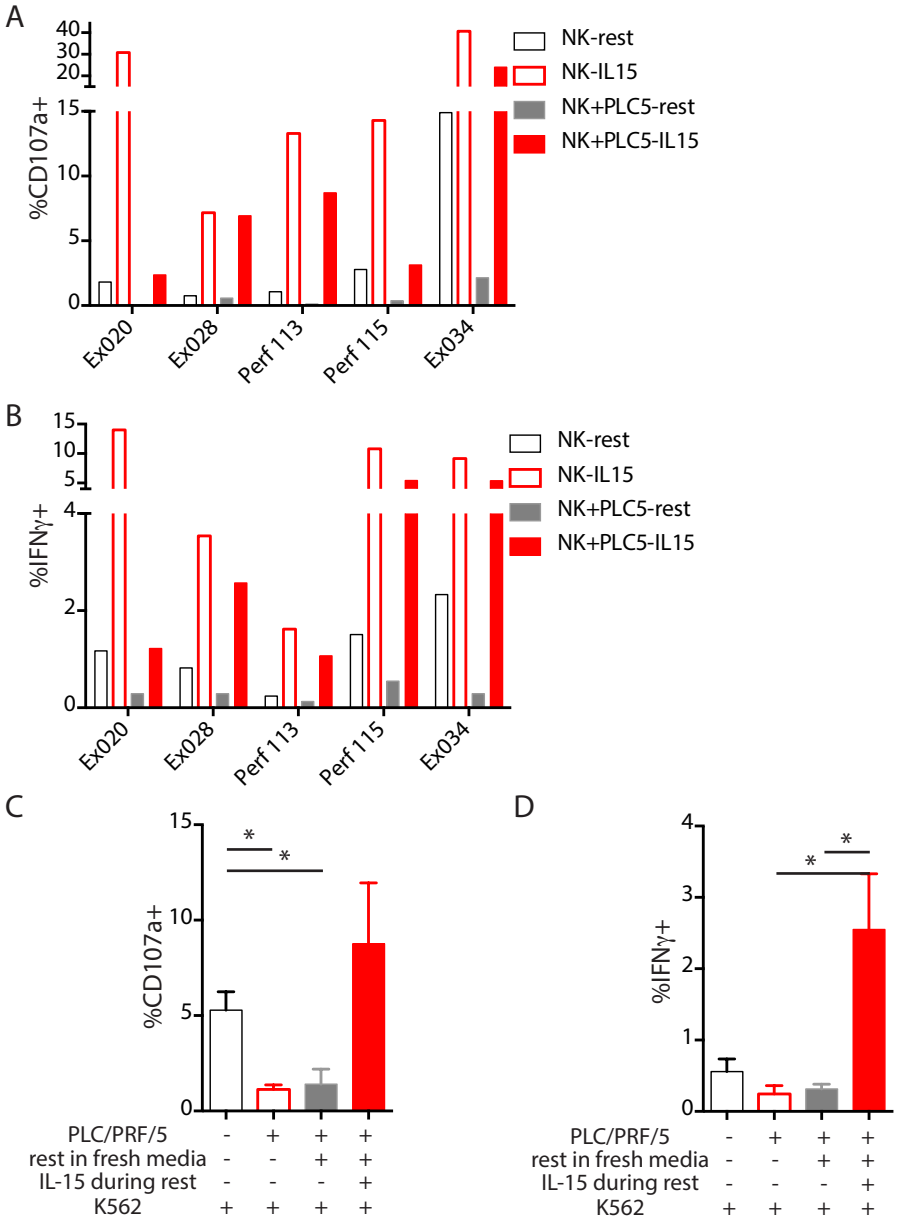

Figure 4.21 IL-15 overcomes PLC/PRF/5 mediated functional inhibition

A. CD107a staining by flow cytometry of intrahepatic NK cells from liver explants and perfusates following K562 challenge after coculture with and without PLC/PRF/5 cells, then rest with and without IL-15.

B. Intracellular interferon γ staining by flow cytometry of intrahepatic NK cells from liver explants and perfusates following K562 challenge after coculture with and without PLC/PRF/5 cells, then rest with and without IL-15.

C. Summary data showing CD107a staining following K562 challenge of intrahepatic NK cells unstimulated, after PLC/PRF/5 coculture, after PLC/PRF/5 coculture then rest in fresh media and after PLC/PRF/5 coculture then rest in IL-15 containing media (n=5).

D. Summary data showing intracellular interferon γ staining following K562 challenge of intrahepatic NK cells unstimulated, after PLC/PRF/5 coculture, after PLC/PRF/5 coculture then rest in fresh media and after PLC/PRF/5 coculture then rest in IL-15 containing media (n=5).

Ex, explant; Perf, perfusate.

Groups were compared using unpaired t test with Welch's correction. P ≤0.05 was considered to be significant for all tests. Figures are labelled: *, p ≤0.05; **, p ≤0.005; ***, p ≤0.001;

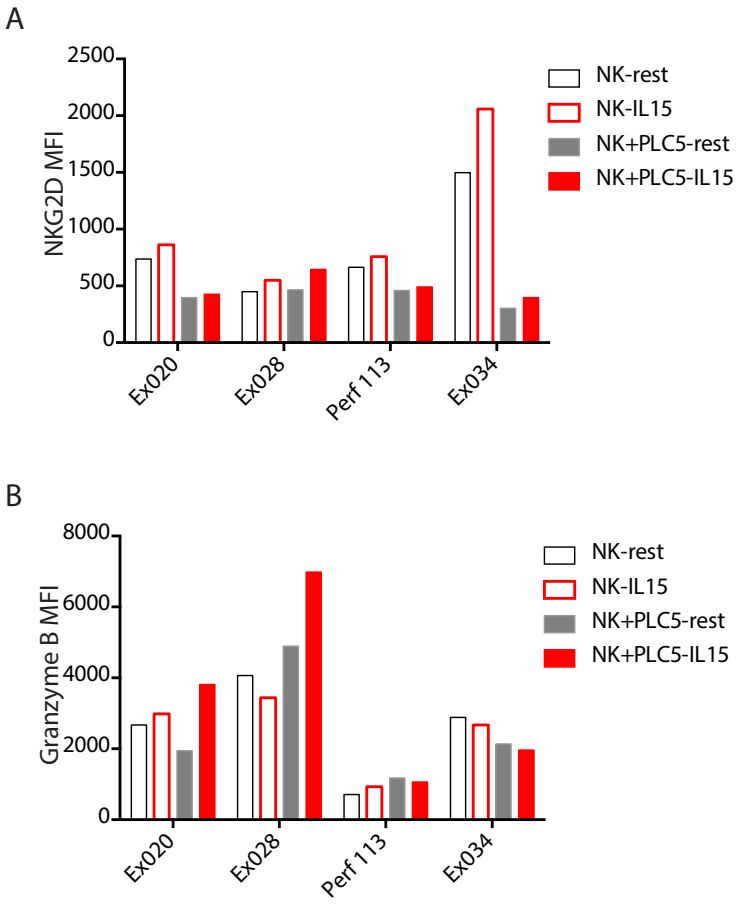

Figure 4.22 IL-15 does not have consistent effects on NKG2D or Granzyme B expression

A. NKG2D expression on intrahepatic NK cells by flow cytometry, expressed as MFI, following K562 challenge after coculture with and without PLC/PRF/5 cells, then rest with and without IL-15.

B. Granzyme B expression on intrahepatic NK cells by flow cytometry, expressed as MFI, following K562 challenge after coculture with and without PLC/PRF/5 cells, then rest with and without IL-15.

Ex, explant; Perf, perfusate.

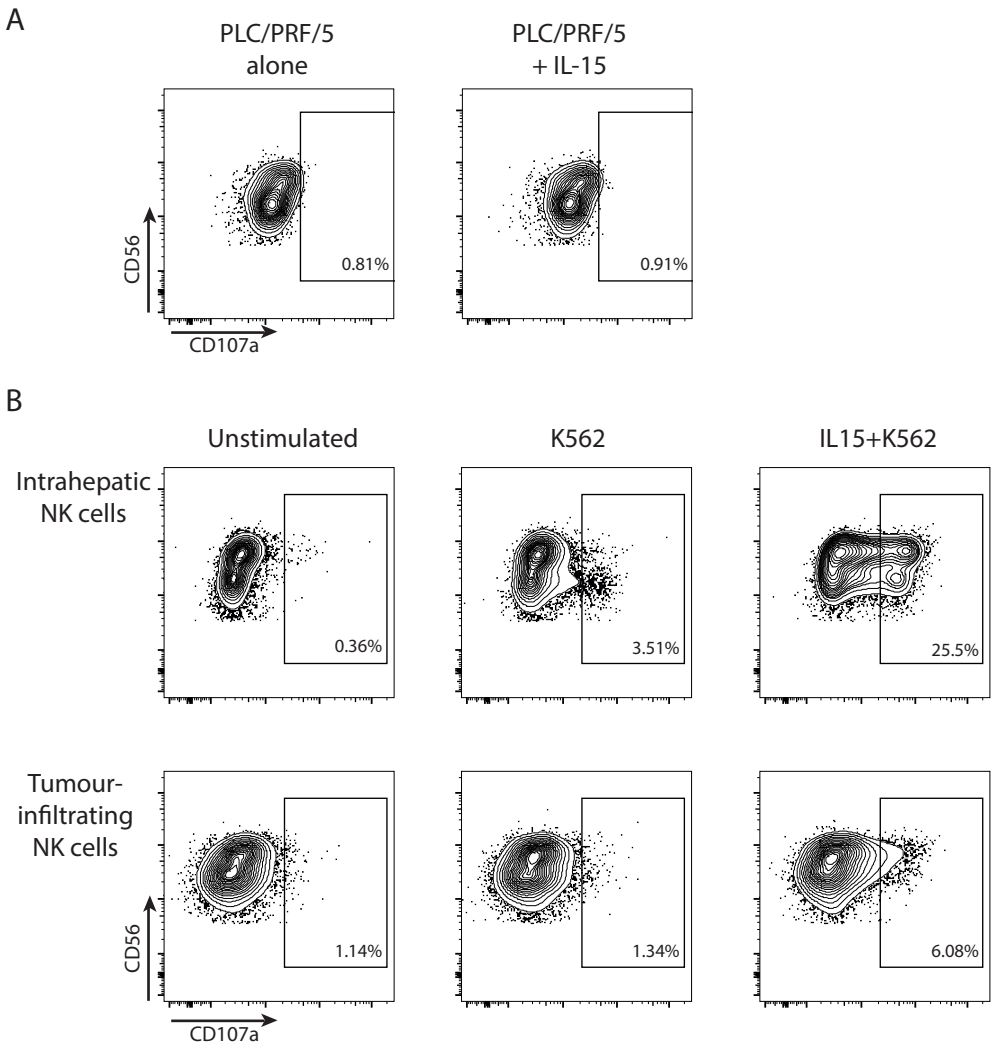

Figure 4.23 IL-15 can restore function of tumour-infiltrating NK cells
A. Example of CD107a staining by flow cytometry on intrahepatic NK cells following overnight PLC/PRF/5 coculture with IL-15.
B. Example of CD107a staining by flow cytometry on paired intrahepatic and tumour infiltrating NK cells following K562 challenge after overnight rest with IL-15.

4.4 Discussion

It is generally considered that CD8 T cells are the most important lymphocyte population and the major anti-tumour effector cells in HCC. This concept appears to have arisen primarily from the work of Wada and colleagues(Wada et al. 1998), who showed in 1998 that a minority of HCC, less than 10%, have profound lymphocytic infiltration. In six of these tumours, which were all moderately differentiated, immunohistochemical staining showed 59% of CD45+ cells were CD8+, 46% were CD4+ and 7.5% were CD20+. These add up to well over 100%, implying no other lineages with significant populations, although no other markers were investigated. Many of the CD8+ cells in the Wada study will have been NKT or non-classical T cells, such as MAIT cells (Kurioka et al. 2016) or Gamma-delta T cells (Bouet-Toussaint et al. 2007; Hoh et al. 2012). Lymphocytes were seen infiltrating the other 90% of HCCs, but the separate populations were not enumerated. Based on these findings CD8 T cells have been the focus of HCC tumour immunology. Our results however show NK cells outnumbering CD8 T cells within liver tumours, suggesting an important role for these innate lymphocytes. Importantly NK cells have anti-tumour activity without a requirement for presentation of and specificity for tumour antigen or neoantigen, thereby offering a large pool of potential effectors.

More recent work has demonstrated a significant NK cell population in HCC (Cai et al. 2008), as in healthy liver tissue (Doherty et al. 1999). Colorectal metastases to the liver are infiltrated by NK cells, in our study to a similar extent as HCC. In one recent publication, NK cell infiltration was associated with improved survival, although in another (smaller) series there was no association (Donadon et al. 2017; Pugh et al. 2014). By comparison colorectal primary tumours have few infiltrating NK cells in most cases (Papanikolaou et al. 2004; Halama et al. 2011). Our work suggests that the

importance of the NK cell infiltrate in colorectal metastases to the liver may have been underestimated.

Recent work by our group and others has confirmed the presence of a liver-resident NK cell population, transcriptionally distinct from peripheral blood NK cells and identified by the expression of CXCR6 and CD69 (Stegmann et al. 2016). The presence of large numbers of CXCR6+ CD69+ NK cells infiltrating both HCC and CRC metastases suggests that the tumour infiltrating NK cells are predominantly derived from the liver rather than from the circulation. There appear to be slightly fewer liver-resident NK cells in HCC than in CRC, which may reflect the increased vascularity of this tumour (Abdullah et al. 2008; Ippolito et al. 2010).

This study confirms the findings of Harmon et al that liver-resident NK cells are NKp46 high, NKG2D high, in keeping with the “immature” phenotype exhibited by these NK cells (Harmon et al. 2016; Stegmann et al. 2016). Although NKp46 expression is maintained in the tumour milieu, NKG2D expression is significantly reduced, which may be indicative of engagement with NKG2D ligands such as MICA or ULBP1 (Kamimura et al. 2012). The ligand for NKp46, recently identified as complement factor P (CFP) (Narni-Mancinelli et al. 2017), is important in bacterial infection but has not been associated with tumours, which may explain the preservation of this receptor on the surface of NK cells. Despite evidence of NKG2D engagement, tumour infiltrating NK cells do not show evidence of activation as HLA-DR levels are unchanged.

The comparative function of intrahepatic and tumour-infiltrating NK cells is little studied. Murine work suggests that colorectal liver metastases may activate the NLRP3 inflammasome and trigger NK cell-mediated killing of tumour cells via Fas-Fas (Dupaul-Chicoine et al. 2015). In humans Cai et al showed in a small series that global HCC-infiltrating NK cells contain reduced

Granzyme B and Perforin compared with global intrahepatic NK cells and showed reduced cytotoxicity (Cai et al. 2008). Recently Sun et al reported that NKG2A was upregulated in HCC-infiltrating NK cells in response to IL-10 and that NKG2A and its ligand HLA-E were negative prognostic markers (C. Sun et al. 2017). However only 10% of tumour-infiltrating NK cells were NKG2A positive and the enrichment in tumours was predominantly in the CD56-dim NK cells. There was little change in the CD56-bright NK cells, of which the CXCR6+ CD69+ liver-resident NK cells are the major population.

In our study we have sought to compare cytotoxic, cytokine-secreting and proliferative function in both liver-resident and non-resident NK cells from paired livers and tumours. Dividing the intrahepatic and tumour-infiltrating NK cells into liver-resident and non-resident, the difference in granzyme B is driven by the liver-resident fraction, with the non-resident cells having a consistently high expression. Non-resident, liver infiltrating NK cells are most similar to (and probably predominantly derived from) peripheral blood NK cells, which also have high levels of granzyme and perforin.

Gamma interferon may have a range of anti-tumour functions, and although it has been difficult to bring it into clinical practice, this immune effector is an important marker of anti-tumour immune responses (Zaidi & Merlino 2011; Parker et al. 2016). Intracellular staining for IFNγ following stimulation with IL-12 and IL-18 demonstrates a marked reduction in the potential for IFNγ production by tumour-infiltrating NK cells, down to almost zero in some examples. This is consistent with findings in other tumours, for example in breast cancer, where more aggressive tumours were associated with increasing impairment of cytokine production (Mamessier et al. 2011). The reductions we observed were similar in liver-resident and non-resident NK cells.

Tumour-infiltrating NK cells, both resident and non-resident, maintain their proliferative potential. That the impairment of function induced by the tumour does not also impair cell division overall is surprising. In both liver and tumour-infiltrating NK cells, there is a marked difference between the Ki67 levels in NKG2D high and NKG2D low NK cells, despite no difference between resident and non-resident NK cells, which may suggest that recent NKG2D engagement, in the context of tumour, impairs proliferation as well as function.

Different cytokines have been show to have different effects on NKG2D expression and proliferation. For example, IL-2, IL-7, IL-12 and IL-15 can upregulate NKG2D (Roberts et al. 2001; Y. P. Park et al. 2011; Lanier 2015), whereas TGFβ, IFNβ1 and IL21 can downregulate NKG2D (Castriconi et al. 2003; Muntasell et al. 2010; Burgess et al. 2006). NK cell proliferation is promoted by IL-12 and IL-18, particularly in combination, and by IL-15 and IL-21 (Lauwerys et al. 1999; Carson, Giri, Lindemann, Linett, Ahdieh, Paxton, Anderson, Eisenmann, Grabstein & Caligiuri 1994b; Parrish-Novak et al. 2000). Given the maintenance of proliferation with reduced NKG2D expression seen in tumour-infiltrating NK cells, it is tempting to speculate that NK cells may be exposed to IL-21 in the tumour micro-environment. IL-21 has been associated with liver damage in CHB and promotes tumour rejection via an NKG2D-dependent mechanism in certain murine cancer models (Q. Pan et al. 2014; Takaki et al. 2005). Its role in human HCC remains uncertain and should be the focus of future study. Alternatively the tumour environment may be dominated by a cytokine such as TGFβ, reducing NKG2D expression overall, with proliferation maintained by exposure of a proportion of NK cells to an upregulatory cytokine such as IL-12 or transpresented IL-15, explaining why the proliferating NK cells also had the highest NKG2D expression.

In an HCC coculture system, intrahepatic NK cells downregulated NKG2D in a contact dependent manner, without evidence of activation, inducing a phenotype similar to that of tumour-infiltrating NK cells. We used PLC/PRF/5 cells (a moderately differentiated HCC cell line previously known as Alexander cells (Daemer et al. 1980)) that express a range of ligands to allow interaction with NK cells as a model. There was a small but consistent reduction in the proportion of CXCR6+ NK cells recovered after co-culture with PLC/PRF/5 cells. This may suggest that the liver-resident NK cells are "stickier" due to adaptations to the tissue niche, such as expression of adhesion molecules. This may contribute to the potential potency of liver-resident NK cells as anti-tumour effectors. Alternatively there may be a genuine loss of CXCR6+ CD69+ NK cells in the coculture due to cell death or alteration of the phenotype in culture. The reduction is similar to the modest reduction in liver-resident cells between liver and tumour tissue seen *ex vivo*. We took this as confirming the validity of the model, but could investigate the fate of CXCR6+ CD69+ NK cells further by sorting CXCR6+ NK cells and assessing their phenotype after co-culture with PLC/PRF/5 cells. We could also use CFSE or cell-trace violet to quantify cell division and adherence to PLC/PRF/5 cells in this system, but without an appropriate *in vivo* comparator the meaning of our findings would be uncertain.

Visualisation of NKG2D by Imagestream demonstrated that surface NKG2D downregulation due to coculture with PLC/PRF/5 cells is due to internalisation of NKG2D. It is unclear why intrahepatic NK cells seem more susceptible to this downregulation than peripheral NK cells, especially as intrahepatic, non-resident NK cells show a small but consistent reduction in NKG2D surface expression. Work by Quatrini et al demonstrated the link between NKG2D internalisation and activatory signalling (Quatrini et al. 2015). However in our system there is NKG2D internalisation without NK cell activation. To investigate this we used phospho-flow to assess downstream signalling. We were able to demonstrate ERK phosphorylation on PLC/PRF/5

engagement, suggesting either that NKG2D signalling is intact but is being over-ridden or negated downstream by an alternative pathway.

To assess the possible effects of receptor alteration caused by PLC/PRF/5 on NK cell function, NK cells were challenged with K562 cells and degranulation and cytokine production were quantified. Reductions in both cytotoxic potential and IFNγ production were consistent and marked. This functional impairment was contact dependent, but was not reversed by blocking NKG2D-NKG2DL interactions or DNAM-1. NKG2D interactions are important for degranulation in response to K562, so it is not possible to assess the effect of NKG2D blocking antibody in this system. These experiments could be improved by use of alternative cell lines as controls, for example using an HCC line that does not express NKG2D ligands to try to isolate the effects of this pathway, for example the KYN-2 cell line is poorly differentiated HCC and known to be ULBP1 negative and could be tested for other NKG2D ligands (Kamimura et al. 2012). Alternatively an insect line could be used to control for coculture in the presence of metabolically active cells without the expression of any ligands for any NK cell receptor (Barber et al. 2004). I would have liked to use PLC/PRF/5 cells with the NKG2D ligands knocked down with siRNA or knocked out using CRISPR-Cas9 or an alternative method to finally confirm that NKG2D ligand expression is not responsible for the functional impairment seen. This would be the cleanest test of the hypothesis that the NKG2D pathway is important in the loss of function by tumour-infiltrating NK cells. However blocking NKG2D-NKG2DL interactions by pre-incubating PLC/PRF/5 cells with soluble NKG2D only affected the slightest recovery of NKG2D expression, so it is likely that another mechanism is responsible for both NKG2D downregulation and functional impairment. The coculture system data is supported by the observation that intrahepatic NK cell cytotoxicity is impaired after overnight coculture with ex-vivo HCC tissue. It would be possible to characterise the NKG2D ligands and other NK receptor ligands expressed by the HCC in this

case from stored clinical specimens, and it may be that these markers influence the degree of functional impairment induced, but this data is not currently available.

PLC/PRF/5 cells contain the HLA class I alleles A03:01, A33:03, B42:02 (Bw6 group), B53:01 (Bw4 group), C04:01 (C2 group) and C17:01 (C2 group), providing multiple ligands for KIR molecules of both the A and B haplotypes, so HLA blockade is important to demonstrate that inhibitory KIR are not the cause of lost NK cell function, and had no effect on degranulation. This is supported by one example of impaired NK cell degranulation after overnight culture with autologous HCC.

TGFβ and IL-6 are cytokines associated with liver and HCC respectively (Dooley & Dijke 2011; Schmidt-Arras & Rose-John 2016), and both have been reported to impair NK cell function (Viel et al. 2016; Crane et al. 2010; Vredevoe et al. 2004; Cifaldi et al. 2015), so we investigated the effect of blocking these cytokines individually and in combination. However, consistent with the requirement for contact, there was no effect of blocking the IL-6 receptor and/or TGFβ receptor II. In a sense this is unsurprising, as in HCC these cytokines are thought to be produced primarily by non-parenchymal cells such as Kupffer cells, for which there is no equivalent in our model.

Rather than trying to inhibit the functional impairment induced by PLC/PRF/5 coculture, we attempted to reverse the anergic state induced in the cocultured, tumour-infiltrating NK-like, NK cells. NK cells removed from coculture were washed and incubated in fresh medium containing a panel of cytokines. Only IL-15 was able to restore NK cell cytotoxic capacity. IFNγ production was recovered by varying amounts by all the cytokines tested, but IL-15 showed the most dramatic increase. Notably, overnight rest in fresh media did not restore degranulation or IFNγ production, suggesting that functional impairment was not due to a reversible metabolic restriction or

competition for nutrients in coculture. This recovery in degranulation and IFNγ production was consistent, recovering function even when coculture had rendered the NK cells almost totally anergic.

Finally, we have demonstrated that *ex vivo* tumour-infiltrating NK cells can be re-activated by IL-15. This suggests that an IL-15 signal delivered into the tumour might activate tumour-infiltrating NK cells to kill tumour cells and control tumour growth and progression. IL-15 is being used to maintain *ex vivo* activated NK cells following infusion in the context of haematological malignancy and solid tumours in clinical trials (NCT01875601, NCT01385423) (Guillerey et al. 2016). Similar approaches with modified IL-15/IL-15Rα complexes (IL-15 superantigen) are being developed for use in clinical trials (Rautela & Huntington 2017; Rosario et al. 2016). However administration of IL-15 and associated molecules can cause significant toxicity (Conlon et al. 2015), which appears in animal models to be related to systemic production of IFNγ by NK cells (Guo et al. 2015). IL-15 can be targeted to cancer cells by fusion with specific antibodies in order to enhance anti-tumour activity in animal models (B. Liu et al. 2016). It may be that a similar approach could be used in liver tumours to activate tumour-infiltrating NK cells *in vivo* while minimising systemic toxicity.

Chapter 5 Conclusions

We have shown that soluble ULBP1, which can be produced by HCC, is a marker of active CHB and also a prognostic marker in HBV-associated HCC in The Gambia. In the CHB setting, ULBP1 correlates significantly but weakly with HBV DNA viral load. This suggests that ULBP1 production may arise from a process several steps removed from HBV replication, such as hepatocyte clonal proliferation. This idea is supported by the presence of ULBP1 in HCC of other causes, demonstrating that other upstream processes that converge on HCC can also generate soluble ULBP1. We also find that ULBP1 levels may be of independent prognostic significance in HCC, suggesting that ULBP1 has a distinct biological relevance in this setting.

A number of questions arise from these observations:

1. What is the mechanism by which ULBP1 production is triggered in CHB and HCC?

2. Can ULBP1 be used in clinical management of CHB, either as a marker of disease activity or as a tool for screening for HCC?

3. Can ULBP1 be used in prognostication of early stage tumours as well as the very late stage patients seen in The Gambia.

4. Is ULBP1 truly inert, or does it have a biological role in HCC?

The mechanism by which ULBP1 is produced remains unclear. The serum concentration is associated weakly (possibly indirectly) with viral load but not ALT in CHB, and appears to be released by HCC of various aetiologies and also from primary human hepatocyte cultures irrespective of HBV infection. It may be a general property of hepatocyte stress; examination of ULBP1 levels

in a range of other liver diseases (autoimmune, toxic and ischaemic hepatitis, as well as acute viral infection) may shed light on this. Primary human hepatocyte cultures are the only *in vitro* system where we were able to see ULBP1 release. Manipulation of this system will allow us to test different stimuli for ULBP1 induction such as oxidative stress, translocation of bacterial products, DNA damage, to examine the dependence of ULBP1 release on proteases as has been shown for MICA, and to investigate the dynamics of ULBP1 release. ULBP1 has been shown to have a short half life, which may contribute to the variation seen in the patient groups (Fernandez-Messina et al. 2016).

In rich country settings, management of CHB is well established. Treatment is based on assessment of fibrosis, ALT and HBV viral load, which represent prior liver damage, ongoing hepatitis and ongoing viral replication respectively. Liver ultrasound is the first-line screening test for HCC. The safety and efficacy of the newer nucleos(t)ide drugs makes it tempting to consider treatment in patient groups where this has not traditionally been offered (Seto & Yuen 2016), although clinical trials will be required to support this idea, and current clinical guidelines remain unchanged (European Association for the Study of the Liver 2017). ULBP1, which tends to be lower in young individuals, might mark the beginnings of harmful liver pathology that would benefit from treatment. Long clinical trials would be required to establish this, but if ULBP1 is associated with abnormal hepatocyte clonal proliferation, which could be established relatively quickly, it might provide a simple, non-invasive way to monitor liver disease in the early stages of chronic infection. It may also be able to replace AFP in HCC screening, as ULBP1 appears to have a high specificity for HCC compared with other liver masses.

In low-middle income countries, management of CHB is challenging because non-invasive assessment of fibrosis and quantitative HBV viral load

measurement are expensive and widely unavailable (Okonkwo & Onyekwere 2016; Allain & Opare-Sem 2016). ULBP1 measurement could be used as an alternative to these tests to guide both treatment initiation and response. Currently ULBP1 concentration is measured by ELISA, requiring stocks of antibodies and a plate reader. However, if ULBP1 was shown to be a useful alternative to HBV DNA quantitation, a lateral flow assay could be developed or even incorporated into the current HBsAg point of care test for rapid clinical assessment. ULBP1 testing may not distinguish well between active CHB and early HCC, so depending on the setting liver ultrasound might still be desirable.

Prognostication of late stage disease is an important proof of concept, but distinguishing between 20 day survival and 70 day survival is not necessarily very clinically useful, especially in a disease and setting where the possibilities for intervention are so limited. However, prognostication in early HCC, both in Africa and Europe, might be very useful. Having already tested the serum of 71 patients at the Royal Free Hospital it would be simple, with the appropriate research ethics approvals, to contact their GPs to ascertain dates of death to investigate whether serum ULBP1 is prognostic in a UK cohort of HCC of different causes. This may be confounded by treatment (sorafenib, TACE, radiofrequency ablation), but subgroup analysis, probably requiring an expanded cohort would be able to account for this. Similarly a larger investigation of ULBP1 levels in Gambian HCC patients (or in another patient group) would provide the statistical power required to definitively answer the question of whether ULBP1 provides independent prognostic information that is not captured by markers such as bilirubin and albumin. Because of the co-variation of these parameters, a cohort larger than the Prolifica HC4 cohort may be required to establish this.

It appears that soluble ULBP1 is predominantly present as soluble protein and does not cause NKG2D internalisation. However presumably ULBP1 is

released from HCC cells, where it is expressed on the cell surface(Kamimura et al. 2012). It is still unclear whether this has a direct effect on tumour-infiltrating NK cells. I wanted to compare NKG2D expression between tumour-infiltrating and intrahepatic NK cells between ULBP1 expressing and ULBP1 negative tumours. Although NKG2D MFI is consistently reduced on tumour-infiltrating NK cells, in some tumours NKG2D expression is markedly reduced, and it may be that this is a direct result of interaction with HCC via ULBP1. It is possible to stain HCC for ULBP1 by immunohistochemistry (Fig 5.1), however most HCCs are ULBP1 positive, so intrahepatic and tumour-infiltrating NK cells must be analysed from a large cohort of HCC patients to answer this question.

The functional impairment of NK cells in tumours has been observed in a number of settings, and is believed to be a product of the tumour environment, as in certain haematological malignancies comprising individual cells, particularly AML, NK cells can retain activity and have demonstrated clinical effectiveness. We have developed a model system using human cells that recapitulates the inactivation seen in *ex vivo* HCC-infiltrating NK cells and allows for examination of the mechanisms of this inactivation and the testing of methods of reversal of NK cell functional impairment.

The mechanism of NK cell anergy remains unclear, and may be multi-factorial. It is a contact dependent process, which results in durable inhibition of degranulation and cytokine production, which affects both liver resident and non-resident intrahepatic NK cells and can be reversed or overcome by the administration of IL-15. It is difficult to guess what this process might be, and an unbiased tool such as transcriptomic or proteomic comparison of unstimulated and PLC/PRF/5 exposed NK cells (or *ex vivo*, FACS sorted intrahepatic and tumour infiltrating NK cells) would be useful to examine the changes induced by the tumour environment. Recent attempts to apply

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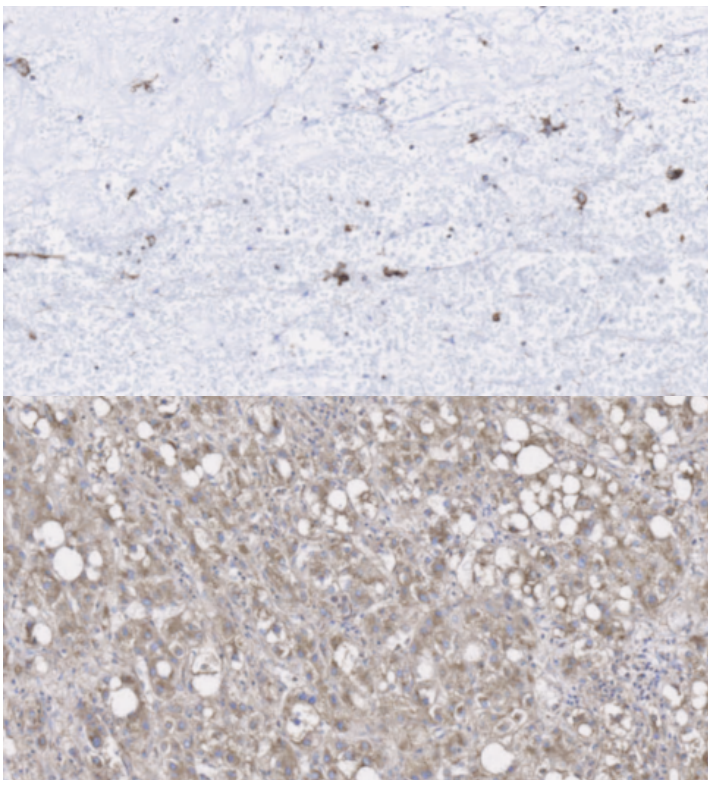

ULBP1 low
colorectal metastasis

ULBP1 high
colorectal metastasis

Figure 5.1 Immunohistochemistry of ULBP1
A. ULBP1 staining by immunohistochemistry of two colorectal metastases, showing low and high ULBP1 expression.
With thanks to Emily Colbeck

transcriptomics to HCC using unsorted cells suggested IL-10 expression by Tregs might contribute to NK cell anergy, but did not suggest a mechanism by which NK cells can be directly inhibited by HCC (Chew et al. 2017). Sorted NK cells might provide cleaner data to examine this question. Proteomic analysis of CD8 T cells has shown that that transcript copy number does not necessarily equate to protein content (Hukelmann et al. 2016), and we might expect NK cells to behave similarly (Bezman et al. 2012). However several million cells are required for this methodology, which may be impractical (by magnetic beads I was able to isolate at most 4 million intrahepatic NK cells and 2 million tumour-infiltrating NK cells from a single explant).

In the absence of a mechanistic explanation for the NK cell anergy seen, the recovery of function with IL-15 has potential clinical applications. Systemic administration of IL-15 is toxic (Conlon et al. 2015), but lower doses, either of IL-15 or IL-15/IL-15Rα superantigen to mimic transpresentation, might be administered intravascularly, directly into the tumour in a manner similar to TACE. To enhance antitumour activity, a bi- or tri-specific protein that is able to induce an immunological synapse between NK cells and HCC targets and also contain IL-15 activity might activate endogenous NK cells to generate a persistent anti-tumour response. Such a strategy has been tested in a mouse model of leukaemia (Vallera et al. 2016). This example used CD16 to target IL-15 to NK cells, but as liver-resident NK cells are CD16 low/negative, alternative markers such as NKG2D, or NKp46, which is high on liver resident NK cells and maintained in the tumour environment, might be used. NKG2D ligands are probably not specific enough for targeting activated NK cells to tumour, instead a specific, cell surface expressed marker of HCC must be used. Many of the promising HCC-specific tumour markers are intracellular proteins (NY-ESO, MAGE-1), but a molecule such as glypican 3 might be a potential target (Shirakawa et al. 2009), or in certain circumstances HBsAg could potentially be used (Bohne et al. 2008; Krebs et

al. 2013). Our culture system with *ex vivo* intrahepatic NK cells, whose anti-tumour responses may be different to and more biologically relevant than those of circulating NK cells, might be a good platform in which to screen candidate therapeutics before progressing to animal models or clinical trials.

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